Identification of *Arabidopsis thaliana* genes that can increase resistance towards phloem feeding insects

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Chapter 1

General introduction

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Interaction between plants and phloem feeding insects

Phloem feeding insects, such as aphids and whiteflies, use visual and/or olfactory cues to locate their host plant (Powell et al., 2006). Upon landing, they use the plant surface features, such as wax layers and leaf trichomes, as the first cues to determine the host plant suitability (Walling, 2008). Then, they use their highly specialized mouthparts (stylets) to intercellularly probe plant tissue and finally reach the phloem (Kaloshian and Walling, 2005) Figure 1). During the first few test probes the insects further evaluate the acceptability of the phloem sap based on, for instance, the nutritional quality (Harris and Kloft, 1992). Once they have established a feeding site, the insects can continue feeding for a prolonged period of time from the phloem (Halarewicz and Gabryś, 2012). During penetration and phloem feeding these insects continuously secrete saliva into the plant (Tjallingii, 2006). Phloem feeding insects excrete a gelling saliva, which forms a sheath around the stylets, to support the intercellular penetration (Miles, 1999). They also excrete watery saliva to degrade cell walls and overcome the occlusion of the feeding site (Will et al., 2009; Moreno et al., 2011). Probing and feeding behavior of the phloem feeding insects can be monitored using the Electrical Penetration Graph (EPG) technique. In EPG the insect, attached to a gold wire, and the testing plant are wired in a low-voltage circuit connected to a recording system (Tjallingii et al., 2010). The recorded signal waveforms are distinguished to represent series of insects' activities (Tjallingii et al., 2010).

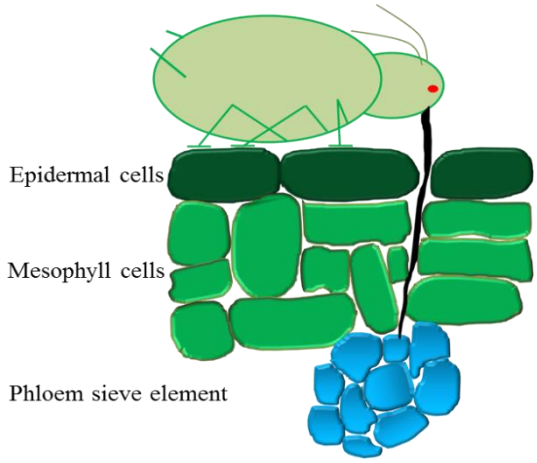

Figure 1. Phloem feeding insect (aphid) uses its stylet to intercellular probe plant tissue and feed from phloem sieve element.

Virus transmission by phloem feeding insects

Phloem feeding insects are important vectors of numerous plant viruses that can be transmitted during probing and feeding by the insects (Weintraub and Beanland, 2006). The viruses can be transmitted in a non-persistent non-circulative or persistent circulative way (Hogenhout et al., 2008). In case of non-persistently transmitted non-circulative viruses, like the potyviruses, the insect acquires the virus on its stylet after a brief probe in an epidermal cell of a virus-infected plant. Subsequent probing on other (healthy) plants will transmit the virus from the stylet to the plant (Pirone and Blanc, 1996). The viruses do not circulate inside the insect body but can be re-acquired on the insects' stylet numerous times. Conversely, viruses that are transmitted in a persistently circulative way, like members of the *Luteoviridae* family, are located in the phloem of the plant (Hogenhout et al., 2008). For acquiring this kind of virus the insect needs to feed for a prolonged period of time (at least 10 minutes) from the phloem sap of infected plants (Hogenhout et al., 2008). The virus particles, taken up with the phloem sap during insect feeding, circulate from the digestive tract, 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). Once acquired, the virus is maintained in the insect for its entire

lifespan, is passed through to the nymphs and can be transferred to every new plant the insects visit (Hogenhout et al., 2008).

Impact of phloem feeding insects on agriculture

Phloem feeding insects are causing more and more damage to agriculture worldwide. These insects deplete the host plant from photo-assimilates and cause chlorosis, stunted plant growth and eventually a reduction in yield (Goggin, 2007; Pompon et al., 2011); Figure 2A). Moreover, phloem feeding insects deposit excess sugars as honeydew that encourages the growth of sooty mould. Mould developed on the plant surface prevents plant tissue from receiving light, further reducing the photosynthetic potential of plants (Wood et al., 1988; Sandström and Moran, 1999); Figure 2B). Phloem feeding insects also pose a threat to agriculture by vectoring numerous plant viruses, which can have devastating effects on food production (Kang et al., 2005; Stafford et al., 2012) Figure 2C and D). Among the 697 virus species recognized by the international Committee on Taxonomy of Viruses (ICTV), aphids and whiteflies transmit 46% (Hogenhout et al., 2008).

To date, the main way to control phloem feeding insects is the frequent use of insecticides, which is only partly successful and on top of that hazardous to the environment (Bass and Field, 2011). Insecticides are also harmful to beneficial insects like pollinators and natural enemies (Lewis et al., 1997; Lewis et al., 1997). Therefore, alternative control methods are needed. Biological control is applied by using natural enemies of the pest insects but the suppression of population development of the pest insects is weak (Kindlmann and Dixon, 1999), especially under field conditions. Thus, the use of resistant varieties would be a more environmental friendly and sustainable solution.

Figure 2. Damages due to infestation of phloem feeding insects. A. Heavy infestation of aphids on cabbage (www.omafra.gov.on.ca), B. Sooty mould covering leaves (apps.rhs.org.uk), C. A lettuce infected by viruses (www.dpvweb.net/intro/ Photo: INRA Bordeaux, France), D. A tomato plant infected by viruses. (http://vegetablemdonline.ppath.cornell.edu/ Photo courtesy of T.A. Zitter, Cornell University, Ithaca, NY)

Host plant resistance traits

To defend themselves against attacks of phloem feeding insects plants have evolved series of resistance traits (Howe and Jander, 2008). These plant resistance traits can be based on antixenosis and/or antibiosis. Antixenosis based resistance results in a change in insect preference for the host plant, while (strong) antixenosis affects the physiology of the insect (Smith, 2005). Antixenosis is the

first defense line by preventing insects from landing and settling (Aharoni et al., 2005; Unsicker et al., 2009). To interfere with host selection, plants can produce deterrent volatiles to repel insects. A nice example of repellent volatiles has been shown in the wild potato *Solanum berthaultii*, which releases the sesquiterpene (E)-*β*-farnesene that repels aphids (Gibson and Pickett, 1983). Upon landing, plants may have physical barriers to prevent insects from settling and to cause difficulties in plant penetration (Mauricio and Rausher, 1997; Muigai et al., 2003). For instance, the formation of glandular trichomes on potato and tomato leaves is one of the most effective resistance traits against insects as they are entrapped by the sticky exudates of the trichomes (Wagner et al., 2004; Rodriguez-Lopez et al., 2011).

Antibiosis based resistance results in reduced reproduction and the population development (Smith and Boyko, 2007). During the first few phloem contacts, unfavored phloem sap content such as toxic compounds can lead to inhibited feeding and reduced development of insects (Kliebenstein, 2004). For instance, glucosinolates, a major class of crucifer-specific secondary metabolites and their *β*-thioglucosidases breakdown products deter generalist insects from feeding (Hopkins et al., 2009). Lectins and proteinase inhibitors reduce insect development by interfering with processes in the insect gut and inhibit insect digestive enzymes (Philippe et al., 2007; Carrillo et al., 2011). Methyl ketones, a class of fatty-acid derived volatile compounds, are toxic to several insects (Kennedy, 2003). One of the identified methyl ketones, 2-tridecanone, was shown to be lethal to cotton aphids (Williams et al., 1980). In wild tomato plants, 7-epi-zingiberene mediates a strong repellence and is lethal to whiteflies if no alternatives are provided (Bleeker et al., 2012). Furthermore, acyl sugars function in plant resistance to insects by sticking and immobilizing insects (Wagner et al., 2004). In wild tomato, acyl sugars affect the settling of whiteflies and subsequently reduce the oviposition of the insects (Liedl et al., 1995). Different acyl sugars have different properties that are toxic to a variety of insects (Puterka et al., 2003).

All of the plant resistance traits can either be constitutive or induced upon the attack by phloem feeding insects (Karban et al., 1997). Constitutive resistance serves as the first defense line to resist insect attack while induced defense responses, which are activated upon insect infestation, prevent further damage (Kessler and Baldwin, 2002). Since defenses require energy and reallocation of resources it is cost effective to induce defense upon specific attackers (de Vos, 2006), while constitutive resistance is only cost effective under high insect pressure conditions (Underwood and Rausher, 2002).

Molecular mechanisms underlying plant resistance to phloem feeding insects

Plant resistance against insects can be conferred by resistance (*R*) genes. Plant *R* genes consist of a nucleotide bindings site (NBS) and a leucine-rich repeat (LRR) motif (Kaloshian, 2004). They work according to a gene-for-gene principle in which plant *R* genes recognize the insect derived elicitors and activate an insect specific defense response (Dodds and Rathjen, 2010). Till now only a limited number of *R* genes that confer resistance to phloem feeding insects have been identified (Broekgaarden et al., 2011; Louis et al., 2012). For instance, the *Vat* gene in melon and the *Mi* gene in tomato confer resistance towards melon aphids and a few isolates of potato aphids, respectively (Rossi et al., 1998; Klingler et al., 2001). Several *Bph* genes in rice and *Rag* genes in soybean lead to resistance against brown plant hoppers and soybean aphids, respectively (Du et al., 2009; Zhang et al., 2009; Qiu et al., 2010; Zhang et al., 2010). However, most of the *R* genes conferring plant resistance are easily overcome by the pest insect, like the genes conferring resistance to hessian fly, Russian wheat aphid and Brown plant hopper (Gould, 1998; Sharma et al., 1999; Haley et al., 2004). Up to now, all *R* genes based insect resistances are found in crop plants. To increase the durability of insect resistance, it would be an attractive strategy to combine the resistance mediated by *R* genes with

quantitative resistance traits. Studies on genes conferring quantitative resistance traits have been comprehensively performed in *A. thaliana* plants. The durability of insect resistance can be further enhanced by combining direct defense with indirect ones, such as using natural enemies (Allmann and Baldwin, 2010). Acyl sugars, for instance, involved in direct plant defense (Liedl et al., 1995; Wagner et al., 2004) can also function in indirect defense. Larvae feed on trichomes that produce acyl sugars accumulate high concentrations of ingested and digested acyl sugars, and release a special odor from their bodies that attracts the larvae's natural enemies (Weinhold and Baldwin, 2011).

Plant hormone signaling plays important roles in defense responses against insect infestation (Erb et al., 2012). The three plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the most comprehensively studied in relation to plant defense against insects (Ellis et al., 2002; Mewis et al., 2005; Pegadaraju et al., 2005; Mewis et al., 2006; Pegadaraju et al., 2007; Louis et al., 2012). It has been shown that the SA signaling pathway is often triggered by infestation of phloem feeding insects (Bostock, 2005), while the JA/ET signaling pathway is normally induced by damage of leaf-chewing insects (Thompson and Goggin, 2006). These pathways may interact synergistically or antagonistically with each other to achieve an optimal plant defense response (Robert-Seilaniantz et al., 2011). The cross-talk between SA and JA signaling has led to the proposal of the 'decoy' hypothesis, which states that the host defenses may be manipulated by insects via inappropriate activation of SA signaling, resulting in suppression of the more effective JA signaling (Zarate et al., 2007). The essential role of JA signaling in *A. thaliana* plant defense to insects was demonstrated by the fact that aphid population development was reduced on a mutant that constitutively activates JA signaling and increased on a mutant that blocks JA signaling (Ellis et al., 2002). In contrast, population development on mutants affected in SA signaling is comparable to that on wild type plants (Moran and Thompson, 2001; Pegadaraju, 2005), suggesting that SA signaling does not play a key role in *A. thaliana* defense against insects.

The activation of plant hormone signaling pathways in insect infested plants regulates the expression of specific groups of defense related genes (Robert-Seilaniantz et al., 2011). A large number of activated genes revealed in transcription profile analyses of insect infested plants are involved in cell wall modification, oxidative stress response, water transport, photosynthesis and carbon and nitrogen mobilisation. (Thompson and Goggin, 2006; Broekgaarden et al., 2007; Kempema et al., 2007; Kusnierczyk et al., 2008). Constitutive activation of several individual genes can lead to increased insect resistance, confirming their roles in insect resistance. For instance, constitutive expression of gene *IQ-Domain1* (*IQD1*, At3g09710), gene *MPL1* (lipid biosynthesis related genes *Myzus persicae – induced lipase 1*, At5g14180) and gene *TPS11* (*trehalose-6-phosphate synthase 11*, At2g18700) all led to enhanced aphid resistance in *A. thaliana* (Levy et al., 2005; Louis et al., 2010; Singh et al., 2011).

Arabidopsis thaliana as a model to study plant resistance towards phloem feeding insects

As mentioned above *A. thaliana* has been used as a model plant to study plant-insect interactions (Nishimura and Dangl, 2010; Louis et al., 2012). This is due to several features of *A. thaliana*. First, *A. thaliana* has a short life cycle. Within six months an entire cycle can be completed from seed germination to new seed production and maturation. Second, *A. thaliana* has a small genome that has been completely sequenced and a large number of molecular markers is available (The *Arabidopsis* Genome Initiative 2000). Third, the genome can be manipulated relatively easy, as an efficient transformation protocol and a sophisticated maker selection of progenies is available (Clough and Bent, 1998; Shimada et al., 2010). Furthermore several *A. thaliana* mutant collections have been created (Weigel et al., 2000; Alonso et al., 2003; Radhamony et al., 2005; Dong-Mei et al., 2008). The most well-known ones are T-DNA knockout (Haag, 2007) and activation tag mutants (Weigel et al.,

2000; Marsch-Martinez et al., 2002). Both of them have been used to investigate gene functions (O'Malley and Ecker, 2010). The T-DNA interruption of gene expression generates knockout mutants, while activation tagging uses a T-DNA vector that contains an enhancer to increase the transcription level of genes (Kuromori et al., 2009). The T-DNA knockout mutants, that are available for almost any gene, can be obtained easily from NASC to verify gene function (http://arabidopsis.info/; (Scholl et al., 2000)). With the efficient transformation and progeny selection system it is also rather easy to overexpress the target gene under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter, which is usually used to further confirm the gene function (Aharoni et al., 2004; Levy et al., 2005; Singh et al., 2011). Additionally, there is an advanced annotation of gene function and comprehensive databases providing gene expression profiles of several developmental stages and specific plant tissues as well as the plant responses to numerous (a)biotic stresses (Zimmermann et al., 2004; Goda et al., 2008; Hruz et al., 2008; Peng et al., 2009; Lamesch et al., 2012). This information provides the first insight into the possible function of a gene, which is helpful in designing experiments to reveal the biological function.

Research aim and thesis outline

The aim of my research was to identify genes in *A. thaliana* that can increase resistance to phloem feeding insects. It is hypothesized that genes that may increase insect resistance are already present in plants, but the level to which these genes are expressed is too low or the timing of expression is wrong. Once being overexpressed, these genes may lead to an increased plant resistance against insects. To identify such genes screening of activation tag gain-of-function mutant collections is very helpful. In such collections tagged genes are overexpressed by a strong CaMV 35S enhancer adjacent to the natural promoter, which results in a dominant gain-of-function phenotype (Marsch-Martinez et al., 2002); Figure 3). Wild type *A. thaliana* is susceptible to several phloem feeding insects, including aphids and therefore a suitable plant to identify mutants with increased insect resistance due to increased gene expression. Identified mutants can either directly reveal the gene function or provide clues about the pathway(s) the gene is involved in. Generalist aphid *Myzus persicae* is selected as a model to study plant resistance towards phloem feeding insects, as it does not adapt to specific plant defense responses. Figure 4 outlines the scheme which was followed to come to the results described in the different chapters of this thesis.

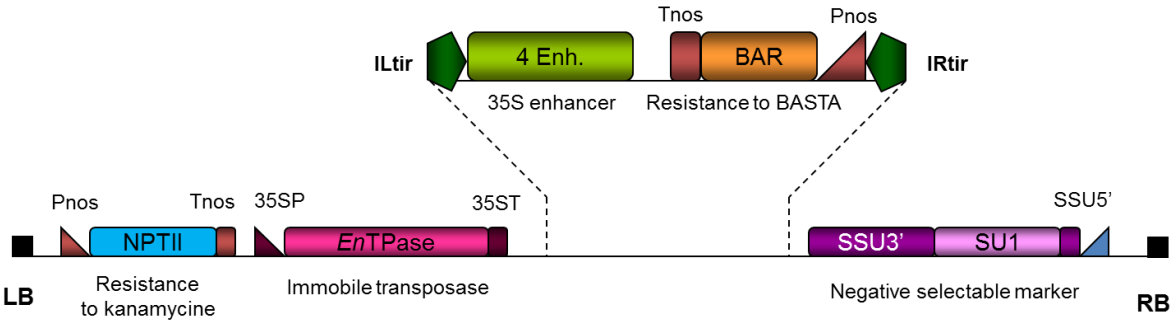

Figure 3. Construct used to increase expression of flanking genes in the activation tag mutant collection. Modified after (Marsch-Martinez et al., 2002).

The activation tag construct consists of three main components: (1) the *En (Spm)* transposase coding sequence under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter and terminator sequences; (2) a mobile, non-autonomous *I* (*dSpm*) component harboring a tetramer of the CaMV 35S enhancer and the *BAR* gene between the terminal-inverted repeats, denominated activating I element (AIE); and (3) the negatively selectable marker *SU1*, adjacent to the transposon components within the

T-DNA. This system uses the selectable markers *BAR* conferring resistance to the herbicide Basta and *SU1* that converts the pro-herbicide R7402 (DuPont, Wilmington, DE) into the herbicide sulfonylurea inhibiting or reducing the growth of plants that contain it.

The construct was introduced by *Agrobacterium tumefaciens*-mediated transformation into *Arabidopsis thaliana*. Activated transposase recognizes the terminal-inverted repeats (ILtir and IRtir) and excises AIE from the T-DNA. The released AIE randomly inserts into plant genome. Within a distance of approximately 8 kb of the CaMV 35S enhancer the expression level of genes may be up regulated.

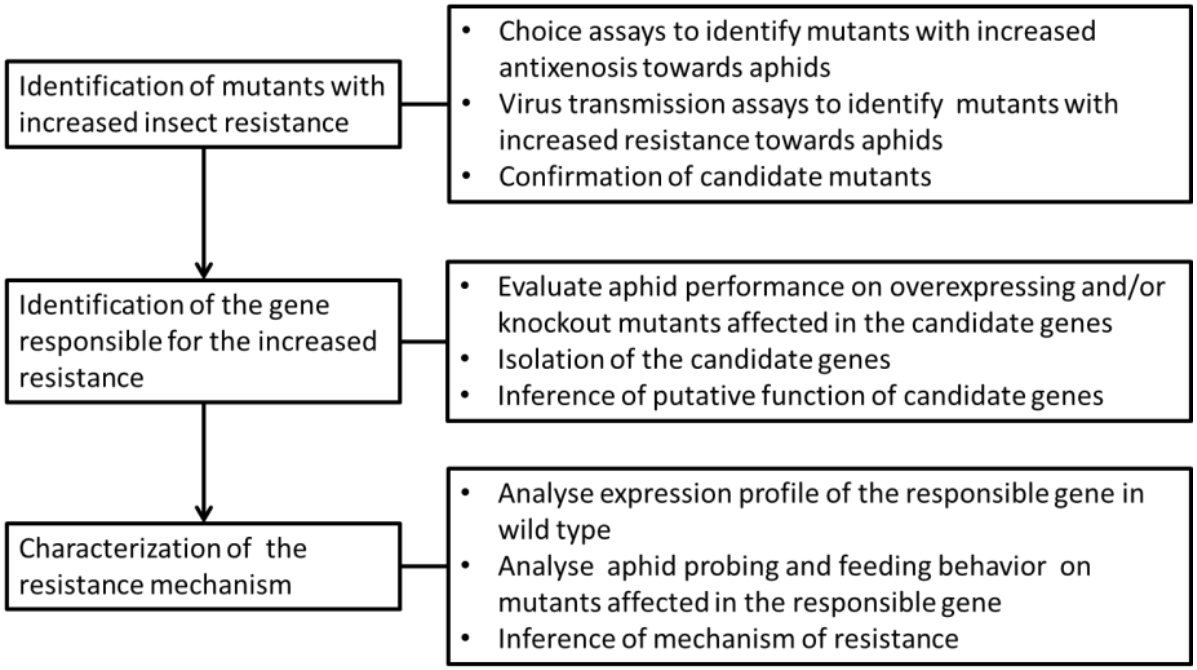

Figure 4. Scheme of the thesis outline.

To identify aphid resistance in *A. thaliana* mutants, we first aimed at antixenosis since it is the only resistance mechanism that can fully prevent the virus transmission by phloem feeding insects. In Chapter 2, we investigated whether it is possible to identify antixenosis based resistance in the activation tag mutant collection. Based on the hypothesis that there is a trade-off between plant fitness and plant resistance, we screened a subset of the activation tag mutant collection. The mutants used were previously selected for their reduced growth fitness to increase the chance of successful identification of resistance. To identify antixenosis we used a series of choice assays and selected one mutant that displayed enhanced antixenosis towards aphids. We showed that the antixenosis is phloem based and requires intact plants. Unfortunately, due to unknown reasons, we did not manage to locate the enhancer in the genome of this mutant and were therefore not able to identify the gene responsible for the enhanced antixenosis.

In Chapter 3, we screened a large number of mutants from the collection for resistance in general (both antixenosis and antibiosis). As introduced above, the transmission of the persistently circulative viruses depends on phloem feeding of the aphid and plant resistant traits may interfere with the insects' ability to reach the phloem. In principle, virus transmission can therefore reflect plant resistance to the insect that vectors the virus. We established a high throughput screening system to identify mutants with increased resistance towards the aphid. We used the persistently circulative *Turnip yellows virus* (TuYV) to indicate enhanced resistance of *A. thaliana* activation tag mutants

against *M. persicae*. Using this method we identified nine mutants with reduced virus transmission in a collection of 5160 mutant lines. These nine candidate mutants were all confirmed to have increased resistance towards aphids thereby showing the reliability of the system.

Further characterization of the mutants revealed the genes that are responsible for the increased aphid resistance as well as the resistance mechanisms involved. In Chapter 4, we characterize one of the aphid resistant mutants, and identified the responsible gene, which we named *Increased Resistance to Myzus persicae 1* (*IRM1*). In wild type plants the expression of *IRM1* is strongest in the xylem and very low in other plant tissues. We show that overexpression of *IRM1* in all tissues of *A. thaliana*, results in a mechanical barrier that makes it difficult for *M. persicae* to reach the phloem. The reduced aphid's capability of reaching the phloem on *IRM1* overexpressing plants probably reduces the transmission of persistent viruses as well.

In Chapter 5, we characterized another mutant with increased aphid resistance for which *SKU5 SIMILAR 13* (*SKS13*) is the responsible gene. In wild type plants gene *SKS13* is exclusively expressed in pollen. We confirmed that it is not expressed in leaves and also that it is not inducible by aphids. Aphid resistance conferred by overexpression of *SKS13* in *A. thaliana* is phloem based; it reduces aphid phloem sap ingestion. The overexpression of *SKS13* leads to an accumulation of Reactive Oxygen Species (ROS) in leaves which may explain the phloem based aphid resistance. The reduced aphid's phloem feeding on *SKS13* overexpressing plants restricts the persistent virus transmission.

In Chapter 6, the results from the experimental chapters are discussed with reference to our present understanding of plant resistance mechanisms towards phloem feeding insects. Furthermore the perspective of using the genes identified in *A. thaliana* in crop plants to increase their insect resistance is discussed.

Chapter 2

Scared off by taste: identification of an *Arabidopsis thaliana* activation tag mutant that repels *Myzus persicae*

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Abstract

The interaction between plants and aphids starts with the selection of a suitable host by the aphid. To prevent aphids from selecting them, plants have evolved antixenosis based defense traits. Antixenosis can affect aphid selection behavior prior and/or after landing through visual, olfactory or taste cues. Here, we describe the identification and characterization of an *Arabidopsis thaliana* activation tag mutant showing enhanced antixenosis based resistance against the green peach aphid *Myzus persicae*. Plant volatiles were not the factor driving antixenosis in this mutant as aphids did not discriminate between volatiles emitted by mutant and wild type plants. When forced on the mutant, aphids performed equally well on mutant and wild type. However, when the aphid was given a choice it preferred to leave the mutant. Using the EPG technique it was shown that the number of phloem salivations and the subsequent phloem sap ingestion differed between mutant and wild type. These results indicate the presence of deterrent factors, leading to an antixenosis based resistance that resides in the phloem.

Key words

plant resistance, antixenosis, choice assays, Y-tube, electrical penetration graph (EPG)

Introduction

Aphids use olfactory and visual cues to locate their host plants. Once on a plant the aphids walk around and detect a variety of cues, like trichomes or volatiles, to determine if they should stay or leave. Subsequently, aphids start probing plant tissues to further evaluate the host plant suitability based on accessibility and quality of the phloem sap (Powell et al., 2006). When the aphid accepts a host plant, it establishes a feeding site and may continuously feed for a prolonged period of time (Tjallingii, 1990). While feeding, aphids do not only take nutrients and photoassimilates from the plant but they also can transmit many types of viruses. One test probe is already sufficient to transmit non-circulative viruses. During the aphid's prolonged feeding process circulative plant viruses may be transmitted as well (Hogenhout et al., 2008). Aphids excrete excessive sugar in the form of honeydew (Taylor et al., 2012), which is a perfect substrate for moulds. Phloem sap consumption, virus transmission and the growth of molds stimulated by honeydew excretion seriously reduces crop yield and quality (Kang et al., 2005). Insecticides are widely applied to control aphids, but environmental concerns resulting from the use of insecticides demand for alternative strategies (Huang et al., 2009). Development of host plant resistance is a more environmental friendly and sustainable solution (Broekgaarden et al., 2011).

Plants have evolved resistance mechanisms to defend themselves against aphids including antixenosis and antibiosis (Helmut van, 2002). Antixenosis serves as the first line of defense, preventing aphids from settling. For example, plants can emit chemical compounds that are volatile to repel aphids (Aharoni et al., 2005; Unsicker et al., 2009). Compounds present in the phloem, such as the plant secondary metabolites glucosinolates and alkaloids, can also play an important role in deterring aphids from feeding (Mndolwa et al., 1984; Kim et al., 2008). When aphids overcome the antixenosis and start to colonize the plant, antibiosis comes into play. Antibiosis increases aphid mortality and reduces fecundity by generating for example toxic compounds or reducing the nutritional quality of the phloem sap (Smith, 2005; Smith and Boyko, 2007). These two plant resistance mechanisms may function complementary to each other.

Plant defenses are costly as they recruit substantial resources away from growth and reproduction (Heil and Baldwin, 2002). Therefore, it is hypothesized that defense trades off with plant fitness, i.e. growth and reproduction (Agrawal et al., 2002; Kempel et al., 2011). This is most likely the reason why plants have evolved induced defenses, i.e. defenses that are only activated in the presence of insects. Constitutive expression of defense related genes in, for example, mutants may result in a reduced growth phenotype (Chapter 4, 5; (Kempel et al., 2011). Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are plant hormone pathways that play important roles in plant defense responses (Robert-Seilaniantz et al., 2011). Constitutive accumulation of SA in the *Arabidopsis thaliana ssi2* mutant, which shows enhanced resistance to aphids, accelerates cell death and dwarf morphology (Sekine et al., 2004; Louis et al., 2010). Constitutive activation of JA and ET in the *A. thaliana cev1* mutant leads to enhanced resistance to several insects and is accompanied by a dwarf phenotype (Turner et al., 2002).

Previously, an *A. thaliana* activation tag mutant collection was screened for mutants showing a reduction in plant fitness (e.g. lower seed set, poorer growth) compared to the wild type (Marsch-Martinez et al., 2002; Dixit, 2008). In these mutants tagged genes are overexpressed by a strong CaMV 35S enhancer adjacent to the natural promoter, which results in a dominant gain-of-function phenotype. The activation tag sequences in these mutants can be used to obtain fragments of the flanking DNA. Based on the sequence of the flanking DNA the position of the insertion can be determined using bioinformatic tools (Altschul et al., 1990) and first indications on the function of the

candidate genes can be obtained from information in a series of databases (Hruz et al., 2008; Lamesch et al., 2012). From the pre-selected *A. thaliana* activation tag mutant collection, in which all mutants show reduced fitness compared to the wild type, we aimed to identify mutants with an increased antixenosis based aphid resistance. Based on the trade-off principle one would expect that in this collection the percentage of mutants with a resistant phenotype will be higher than in a random selection of mutants. Through a series of insect bioassays, one aphid resistant mutant showing increased antixenosis was identified. Comparison of aphid responses to plant odors and aphid feeding behavior between the mutant and wild type suggested that the resistance was not due to plant volatiles but caused by deterrent factors in the phloem.

Materials and Methods

Insect rearing

Myzus persicae was reared in cages on Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis* cv. Granaat). The rearing was maintained in an environment controlled room with a relative humidity of 60-70%; a temperature of 20 ± 2 °C and an 18:6 L:D photoperiod. For all experiments, only apterous aphids were used.

Plant materials and growth conditions

A collection of 170 *Arabidopsis thaliana* mutants, which have an activation tag randomly inserted in the genome of Wassilewskija (WS), was obtained from Wageningen UR Plant Breeding (Marsch-Martinez et al., 2002). Mutants in this collection were pre-selected based on reduced fitness, such as lower seed set and poorer growth (Dixit, 2008). Seeds were vernalized by placing them at 4°C in the dark for 3 days under high humidity. Subsequently, seeds were transferred to rockwool that was fully saturated with Hyponex nutrition solution (Tocquin et al., 2003). Plants were cultivated in a climate chamber, programmed for an 18:6 L:D photoperiod. The temperature was maintained at 20 ± 2 °C during the day and 18 ± 2 °C during the night. The relative humidity was kept at 60-70%. Plants grown on Rockwool were supplemented with Hyponex nutrition solution every two days till they were three weeks old (Tocquin et al., 2003).

Plants for performing electrical penetration graph (EPG) were grown in soil. After vernalization, seeds were transferred to potting compost (Lentse Potgrond®) and plants were watered every other day until they were five-week-old. No additional nutrients or pest control was applied.

Choice assays

Choice assays were conducted in an arena setup, in which a 10 cm-diameter, 2 cm-thinness Styrofoam plate was divided into six identical pie sections to contain six plants. Thirty young adult aphids were placed in the middle of the arena using a fine brush and allowed to choose host plants for 24 hours. Individual arenas were separated by water to prevent aphids from crossing between arenas. After 24 hours, the number of aphids on each plant was recorded.

In the first choice tests, one arena was constituted with six different plants: five different mutant lines and one wild type (Figure 1A). Each arena was independently repeated five times in the same arrangement. Data were analyzed within each arena by dividing the number of aphids on each plant by the total number of aphids on all six plants in that arena. Values were square root transformed and subsequently used to determine significance between mutant and wild type by ANOVA followed by the Tukey tests ($P < 0.05$).

To retest the candidate mutants identified from the first choice test, we performed a second choice assay. In this test an arena contained three identical mutant plants and three wild type plants, organized in an alternating way (Figure 1B). Fifteen replicates were made for each arena. The frequency of aphids on each genotype was calculated by dividing the number of aphids on mutant or wild type plants by the total number of aphids on all six plants in the arena. The aphid preference was determined by using a χ^2 test with the null-hypothesis that aphids did not have a preference for one of the two genotypes.

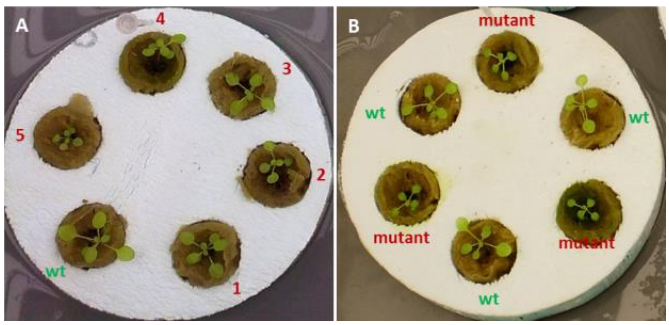

Figure 1. Arena setup of choice assays. (A) In the first choice assays, arenas contained plants of five different mutant lines (numbers 1-5) and one wild type (wt). (B) In the second choice assays, arenas contained three plants of a mutant line and three wild type plants that were organized in an alternating way.

Y-Tube experiment

Aphid response to plant volatiles released by mutant and wild type plants was assessed in a Y-tube experiment (Koschier et al., 2000). Thirty plants were individually placed in a jar connected to an arm of the Y-tube. The plant growth substrate was wrapped with aluminium foil to block contaminant odors. The Y-tube has 30-cm long arms and an 18-cm long base, with a 3.5 cm-inner-diameter. An air flow of 3 cm/second was pumped through activated charcoal and led through the jars containing the odor sources. The Y-tube experiment was carried out at 20 ± 2 ^{o}C from 10.00 to 13.00 hrs, under constant light. Aphids were introduced individually at the base of the Y-tube and allowed to make a choice within 5 minutes between the two arms of the Y-tube. A choice was considered to be made when an aphid moved 5 cm into one arm and remained there for at least 20 seconds. Otherwise, it was considered as no choice had been made. The odor sources were interchanged after testing six aphids and the plants were replaced by new ones after testing 12 aphids. In total 48 aphids were tested. The aphid preference was determined by using a χ^2 test with the null-hypothesis that aphids did not have a preference for one of the two odor sources.

Settling tests

Settling tests were conducted in an arena setup with either intact plants or detached leaves. Ten young adult aphids were directly released on plants using a fine brush. With intact plants, an arena contained six plants (three identical mutant plants and three wild type plants; Figure 1B). To investigate whether aphids settled on the mutant plants, mutants in the arena received aphids and wild type plants did not. To investigate whether aphids settled on the wild type plants, wild type plants in the arena received aphids and mutant plants did not. There were 15 replicated arenas with 30 aphids per arena. In the settling test with detached leaves, six leaves of about the same size were cut from six individual plants (three from mutant and three from wild type) and were placed alternating in the arena with the abaxial side upward on water agar (15g/L) in Petri dishes (Maharijaya et al., 2011). Due to a smaller area of a single leaf compared to an intact plant, only three young adult aphids were directly released on each leaf. If three detached wild type leaves received aphids, then mutant leaves did not; if mutant detached leaves received aphids, then wild type leaves did not. This resulted in nine aphids in total for each detached leaf arena. Aphids were allowed to move freely between plants (or leaves) and the number of aphids on each plant (or leaf) was recorded 0.5, 6 and 24 hrs after the aphids were released. The number of aphids settled on the genotype that they were released on was compared to the number of

aphids settled on the other genotype present in the arena by using Independent-samples *t* test to determine the significance ($P < 0.05$) between genotypes within each time point.

No-choice assays

Synchronized one-day-old nymphs were transferred onto plants using a fine brush. Each plant received one nymph and the total number of aphids was counted 14 days after infestation. Plants were paced in a randomized design with 15 replicates per genotype. Individual plants were separated by a water barrier. Independent-samples *t* test was used to determine if there was a significant ($P< 0.05$) difference between the genotypes.

Electrical penetration graph

The electrical penetration graph (EPG) technique was employed to monitor aphid feeding behavior (Tjallingii, 1990). In the EPG system, a 20 µm-diameter gold wire was attached to the dorsum of a young adult aphid using conductive water-based silver glue (Ponder et al., 2001). Each wired aphid was placed onto a plant that was connected to a recording system via a copper electrode inserted in the soil (Tjallingii, 2006). The EPGs were recorded at 20 ± 2 ^{o}C under constant light for eight hours. The EPG data were analyzed using the PROBE 3.0 software (Wageningen University, the Netherlands). Waveform C represents the pathway phase, when the aphid stylet is penetrating through the leaf tissue; waveform E1 represents phloem salivation; waveform E2 represents phloem sap ingestion; Waveform F is associated with derailed stylet mechanics or penetration difficulties; and waveform G indicates active uptake of water from the xylem elements (Tjallingii, 1990). For each genotype, 15 recordings of individual aphids were obtained with one aphid per plant. Mann–Whitney U tests were used to determine significant differences between genotypes for individual EPG parameters.

Results

Identification of mutant lines with antixenotic effects on aphids

A collection of 170 *A. thaliana* (accession WS) activation tag mutants was previously selected based on reduced fitness (Dixit, 2008). By acknowledging that reduced plant fitness may be the result of a trade-off with (a)biotic stress resistance (Kempel et al., 2011), we screened this collection to identify mutants with enhanced antixenosis based resistance towards aphids. In the first assay we offered the aphid a choice between six possible host plants, i.e. five different mutant plants and one wild type plant (Figure 1A) and allowed to select a suitable host for 24 hrs. Compared to the wild type, four candidate mutants harbored a significant lower frequency of aphids and one candidate mutant harbored significant higher frequency of aphids than wild type plants (ANOVA followed by Tukey tests, $P< 0.05$). To confirm these results, the candidate mutants were re-evaluated in the second choice assays. Essentially the same arena setup was used, but now with three plants of one candidate mutant against three plants of the wild type, which were placed alternating in the arena (Figure 1B). Unfortunately, one candidate mutant could not be evaluated further due to the unavailability of viable seeds. Compared to the wild type, significantly lower numbers of aphids settled on mutant 435 (χ^2=5.53, d.f=1, P = 0.016). The other three candidate mutants harbored the same numbers of aphids as the wild type (Figure 2). Plants of mutant 435 have smaller rosette leaves than the wild type whereas the color of the rosette leaves, time to flowering and the size of flowers and siliques do not differ from wild type plants.

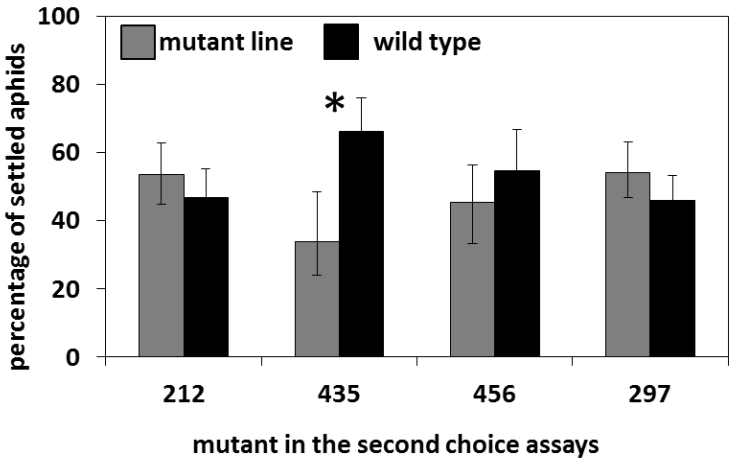

Figure 2. Aphid settling preference between mutant and wild type plants. Values are the means ± standard deviation of 15 biological replicates. The stars indicate significant differences between mutant and wild type plants (χ^2 test, $P < 0.05$).

Characterization of the antixenotic factors in mutant 435

Volatiles are usually the first cues used by aphids to choose a host plant (Powell et al., 2006). To determine whether the aphids did not settle on mutant 435 due to deterrent volatiles, we investigated the response of aphids to plant odors in a Y-tube experiment (Koschier et al., 2000). Similar numbers of aphids were attracted to either plant odor source and, as a consequence, there were no significant preference differences between mutant 435 and wild type plants ($\chi 2 = 0.05$, d.f. = 1, P =0.830).

To further determine which plant traits of mutant 435 prevented aphids from settling we performed a settling test in which aphids were placed on the mutant but had the possibility to leave afterwards. This test investigates the role of plant contact in the aphid's settlement response. As shown in Figure 3, when they were released on intact plants a significantly higher number of aphids left the mutant compared to the wild type. This response is already visible 0.5 hrs after the start of the experiment. The number of aphids that left the mutant was the same after 0.5, 6 and 24 hrs (Independent-samples *t*

test, $P < 0.05$) indicating that once the aphids leave the mutant they did not go back. When aphids were released on detached leaves, the number of aphids that left the leaf was similar between mutant 435 and wild type plants at all time-points tested (Independent-samples *t* test, $P > 0.05$; Figure 3).

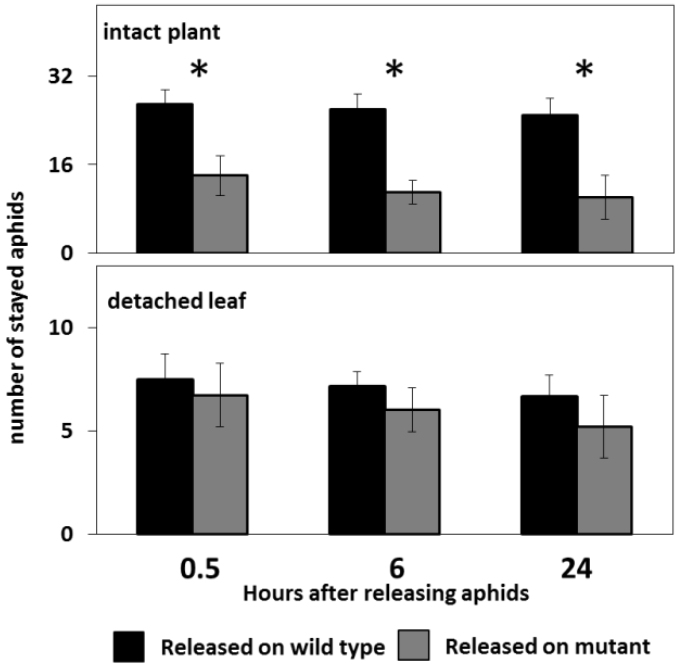

Figure 3. Deterrence of aphids on mutant and wild type. Aphid settling behavior on intact plant and detached leaves of mutant 435 and wild type. Number of aphids present on the plant or leaf was scored at 0.5, 6 and 24 hrs after placing the aphids. Values are the means ± standard deviation of 15 biological replicates. The star indicates a significant difference between bars within a pair at each time point (Independent-samples *t*-test, $P < 0.05$).

Role of antibiotic factors

We investigated aphid performance in no-choice assays to determine whether the mutation in mutant 435 affected aphid fecundity and population development. No significant differences were observed between mutant 435 (15 ± 5 (average ± SD) aphids) and wild type (18 ± 4 (average ± SD) aphids) (Independent-sample *t* test; $P = 0.237$).

Localization of the antixenotic factors

To gain further insight into the location of the resistance factors present in mutant 435, we analyzed the aphid feeding behavior using the electrical penetration graph (EPG) technique (Tjallingii, 1990). All aphids started to penetrate the leaf where they were place on after about the same time, as indicated by the same time to the first probe (Table S1). The EPG parameters associated with pathway phase, xylem phase and derailed stylet mechanics did not differ between the mutant and wild type (Table S1). Moreover, aphids on the two genotypes did not differ in the time from the first probe to the first phloem salivation. Each phloem salivation was followed by phloem sap ingestion and the total time of the phloem sap ingestion between mutant and wild type did not differ (Table S1). Interestingly, significant differences were seen in the number of phloem salivations and the number of phloem sap ingestions (Mann-Whitney U test, d.f. = 30, $P = 0.021$ for both events; Figure 4A) as well as in the average duration of phloem sap ingestion (Mann-Whitney U test, $P = 0.015$; Figure 4B). Representative EPG waveform patterns showed that on all plants (mutant (Figure 5A) and wild type (Figure 5B)) all phloem phases consist of a single phloem salivation event (waveform E1) and a single subsequent phloem sap ingesting event (waveform E2). This indicates that aphids more frequently contact the phloem of the mutant but that the duration of these phloem events is shorter compared to the wild type (Figure 5 A and B).

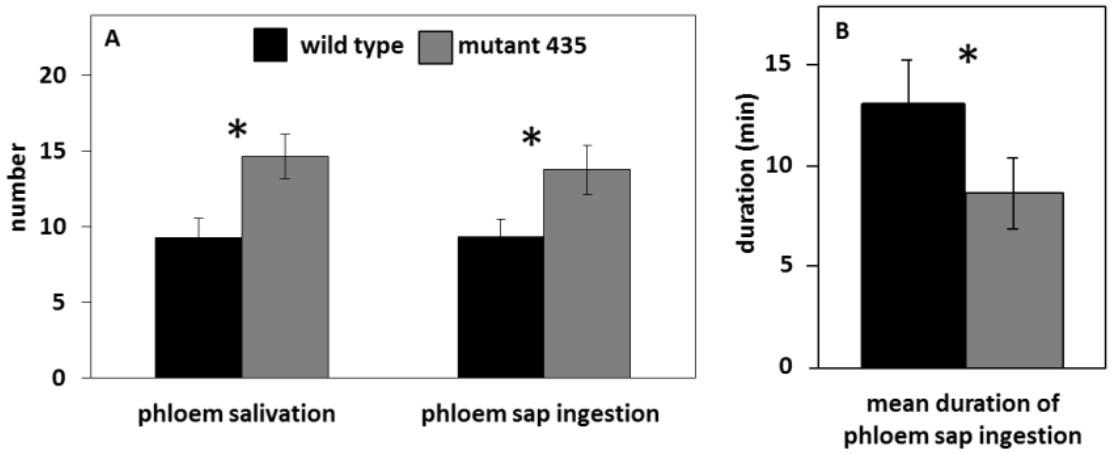

Figure 4. Electrical Penetration Graph (EPG) recordings. (A) The numbers of times that a certain event occurred. (B) Mean duration (min) of phloem sap ingestion The EPG recording with each aphid was conducted for eight hours. Values are means ± SE of 15 replicates. The stars indicate significant differences between bars within a pair (Mann–Whitney U-test was applied for the rest parameters, *$P < 0.05$).

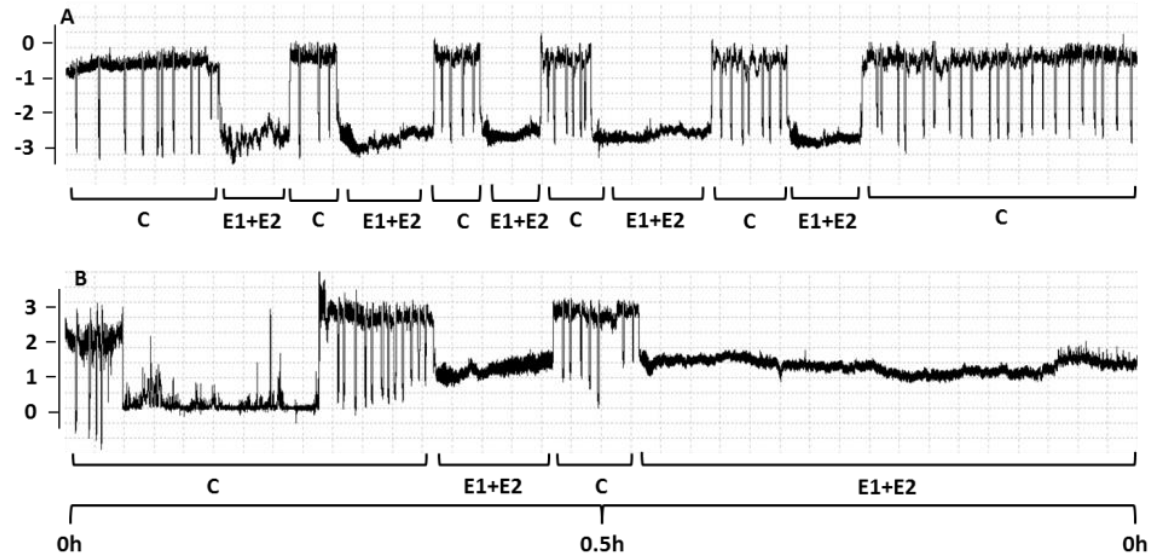

Figure 5. Representative EPG waveform patterns of *Myzus persicae* on mutant and wild type plants. Phloem phase patterns produced when *M. persicae* fed on mutant (A) and wild type (B) plants. The x-axis represents a one hour time period; the y-axis represents voltage. The explanation of the different waveforms is given in the materials and methods section.

Discussion

Screening for antixenosis based resistance towards aphids among *A. thaliana* mutants affected in plant fitness

Plants have evolved several types of resistance to counteract aphid attack. Antixenosis prevents aphids from settling and antibiosis interferes with the life history parameters of the aphid (Powell et al., 2006). Both types of resistance do not only hamper the settling and/or performance of aphids but also reduce the plant's chances of getting infected by viruses. Because antixenosis serves as the first line of defense, this type of resistance is an important factor in the early stage of plant-aphid interactions. In this study we screened 170 *A. thaliana* activation tag mutants for enhanced antixenosis towards the

aphid *M. persicae*. These mutants were pre-selected for reduced growth and seed reproduction (Dixit, 2008) and were expected to show enhanced defenses based on the trade-off principle. After the initial screening, five candidate mutants were identified that attracted significantly less aphids than the wild type and one that attracted significantly more aphids. When these were all retested in a pairwise comparison, only one mutant turned out to be significantly different from the wild type. So, four out of five of our candidates were false positives. The large number of false positives in the initial arena may have been caused by the fact that the aphids' choice for one line may be influenced by the combined effect of the other lines, but this was not analyzed further. The initial arena, in which six lines (five candidate mutants and the wild type) were tested, could serve as a first indication of possible candidate mutants but there is obviously a need to re-test these candidates to finally select the most reliable ones.

The fact that we selected only one candidate mutant out of the 170 from the collection indicates that pre-selecting mutants based on dwarf phenotypes does not lead to the identification of higher percentages of insect resistant plants. It is true that plant defense responses are costly because energy and resources that originally should be used in plant growth and development are devoted into defense (Heil and Baldwin, 2002). This is supported by the fact that many reported resistant mutant plants also display dwarf phenotypes (Turner et al., 2002; Sekine et al., 2004; Louis et al., 2010). Indeed, a reduced growth was observed on the identified mutant in this study as well as on other *A. thaliana* activation tag mutants with enhanced resistance to *M. persicae* that we identified previously (Chen et al. 2012). However, the trade-off principle cannot work the other way around i.e. reduced plant fitness does not necessarily lead to increased resistance. Reduced fitness can result from any impaired functions in plant growth, which would only make plants become weaker than normal grown ones. While in some cases the reduced plant growth is the side effect of plant defense. For instance, reactive oxygen species (ROS) which play a role in plant defense can have serious damage on photosynthesis and ultimately reduce plant growth (Apel and Hirt, 2004; Kerchev et al., 2012). In this sense, the collection of 170 mutants used in this study is a random selection since pre-selection of reduced growth fitness does not help to enhance the percentage of insect resistance.

Antixenosis based resistance of mutant 435 is phloem based and requires intact plants

Plant features, such as deterrent chemical compounds or physical barriers can be the factors of antixenosis based resistance (Gibson and Pickett, 1983; Alvarez et al., 2006). We have shown that the production of plant volatiles that could serve as repellents or attractants are not affected in mutant 435. Also the EPG data revealed no effects on aphids feeding behavior on the plant surface and during the pathway phase. Interestingly, differences were seen in the phloem phase. In comparison to the wild type, aphids showed more frequent phloem salivation and phloem sap ingestions on mutant 435. Also the average length of the periods of phloem sap ingestion was shorter on the mutant.

One possible explanation of the observed differences in the phloem phase may be occlusion of the phloem vessels in response to aphid feeding. Such occlusion may result from forisome dispersion or callose deposition (Kempema et al., 2007; Will et al., 2007; Hao et al., 2008). Forisome dispersion as well as callose deposition are a phloem based defense response that plug the sieve element to interrupt aphid feeding (Tjallingii, 2006). Forisome dispersion causes a typical transition from phloem sap ingestion to phloem salivation, which is believed to function in reversion of the sieve element occlusion (Will et al., 2007). This forisome dispersion is accompanied by a typical EPG pattern, which was not observed for mutant 435 and forisome dispersion can therefore be excluded as the resistance mechanism active in this mutant. Phloem occlusion can also result from callose deposition, which has been interpreted as a defense response as well. In *A. thaliana* the expression of the callose synthase gene and callose accumulation were enhanced in response to whitefly infestation (Kempema et al.,

2007). In rice, callose deposition on the sieve plate is an important resistance mechanism against brown plant hopper (Hao et al., 2008). EPG monitoring of the brown plant hopper feeding behavior on rice varieties revealed that in comparison to susceptible varieties, resistant ones showed a longer period of non-probing, pathway activities and shorter time of phloem sap ingestion (Hao et al., 2008). Moreover, the mean duration of each period of phloem sap ingestion was reduced on resistant varieties (Hao et al., 2008). In mutant 435 we observed similar effect on the duration of phloem sap ingestion, but the prolonged duration of non-probing and pathway activities were not observed. Therefore, it is not likely that callose deposition is the mechanism resulting in the enhanced antixenosis based resistance in mutant 435.

The pattern of aphid feeding behavior of mutant 435 shows striking similarities, e.g. shorter periods of phloem sap ingestion, with patterns observed on plants containing deterrent compounds, such as glucosinolates, in their phloem (Mndolwa et al., 1984; Kim et al., 2008). Glucosinolates affect *M. persicae* feeding by stimulating stylet withdrawal (Byers, 2008), resulting in the reduced duration of each phloem sap ingestion. However, the deterrence by glucosinolates is a local response of plants to resist aphid feeding (Kim and Jander, 2007), and therefore the response was expected to be seen in detached leaves as well. However, this was not the case for mutant 435 as there were differences in aphid settlement behavior between intact plants and detached leaves. Mutant 435 is assumed to be an activation tag mutant, in which the affected gene is overexpressed in every cell ((Aharoni et al., 2004; Pereira et al., 2007), Chapter 4, 5). Therefore the presence of the deterrent compounds in detached leaves should not differ from that in intact plants. An explanation may be that there is a dosage effect of the compound(s) (Cho et al., 2011). In the intact plants, the compound(s) can be transported to the aphid feeding site, accumulated in concentrations high enough to show the deterrent effect on aphids. Conversely, such systemic supply of the compound(s) may be cut off in detached leaves and the amount of local compounds is too low to display the effect on aphids. However, the reason why aphids were deterred only on intact mutant plants and not by detached leaves of mutant 435 remains unclear. First of all, to obtain information on the gene affected in mutant 435, the T-DNA insertion site should be determined. Unfortunately, we were not able to locate the position of the enhancer in the genome of this mutant. This may be caused by the rearrangement or duplication of the T-DNA insertion in this mutant (Tax and Vernon, 2001). Sequencing the whole genomic DNA of mutant 435 is needed to identify the affected gene. Further analyses on the responsible gene and compounds in phloem sap of mutant 435 are needed to gain insight into the nature of the antixenosis based resistance in this mutant.

In the no-choice situation aphid population developed on mutant 435 was as good as on the wild type. From this it can be concluded that the observed antixenosis is not a side effect of antibiosis. Such side effects may, for instance, result from difficulties in accessing the phloem or unfavourable nutritional quality of the phloem (Goggin, 2007).

Conclusion

We have identified an *A. thaliana* activation tag mutant with enhanced antixenosis based resistance to the aphid *M. persicea*. The resistance factor is located in the phloem and requires intact plants in order to be effective. Further research is needed to uncover the molecular basis of this antixenosis based resistance.

Acknowledgements

We thank Unifarm for taking care of the plants; Paul Piron and Greet Steenhuis-Broers for providing and looking after aphid rearing and Dr. Roeland Voorrips for suggestions on statistics.

Table S1. Electrical penetration graph (EPG) results.

		WS			435			P^1
Behavioral variable	#	15			15			
Total duration of non-probing		147	±	24.3	121	±	19.4	0.421
time to the first probe	min	2.9	±	0.9	3.6	±	1.1	0.830
Number of probes to the first phloem salivation	#	5.5	±	2.6	7.8	±	3.5	0.784
Duration of the shortest pathway activity before phloem salivation	min	6.0	±	2.8	7.6	±	2.6	0.871
Total duration of pathway activities		215.3	±	37.2	265.3	±	51.3	0.426
Number of derailed stylet mechanics	#	0.4	±	0.1	0.6	±	0.3	0.654
Mean duration of derailed stylet mechanics	min	3.2	±	2.8	3.7	±	2.6	0.913
Total duration of derailed stylet mechanics	min	3.9	±	2.3	4.8	±	3.7	0.727
Time from first probe to first phloem salivation	min	38.5	±	9.0	44.3	±	11.1	0.371
Time from first probe to first phloem consumption	min	41.3	±	14.5	46.2	±	17.0	0.798
Time from first probe to first sustained phloem consumption (> 10 min)	min	111.5	±	28.9	186.5	±	36.5	0.089
Number of phloem salivation events	#	9.3	±	1.3	14.7	±	1.5	0.021
Mean duration of phloem salivation	min	0.9	±	0.1	0.7	±	0.1	0.364
Total duration of phloem salivation	min	10.8	±	2.4	8.9	±	1.3	0.154
Number of phloem consumption events	#	9.4	±	1.1	13.8	±	1.6	0.021
Mean duration of phloem consumption	min	12.9	±	2.1	8.6	±	2.3	0.015
Total duration of phloem consumption	min	134.0	±	13.8	156.5	±	25.2	0.514
Number of sustained phloem consumption (>10 min)		4.2	±	1.1	5.3	±	1.2	0.456
Mean duration of sustained phloem consumption (>10 min)	min	28.3	±	7.5	19.9	±	11.2	0.156
Total duration of sustained phloem consumption (>10 min)	min	121.3	±	13.4	78.4	±	11.6	0.242
Number of xylem sap consumption events	#	1.2	±	0.4	0.7	±	0.2	0.5
Mean duration of xylem consumption	min	13.6	±	4.2	15.2	±	7.1	0.786
Duration of xylem consumption	min	18.2	±	6.5	14.8	±	6.3	0.815

EPG recording with each aphid was conducted for eight h. Values are means ± SE of EPG parameters. Mann- Whitney U test was used to determine the significant difference between the activities of aphids on the mutant and the wild type plants. Gray boxes *P* value significant different ($P < 0.05$)

Chapter 3

High throughput phenotyping for aphid resistance in large plant collections

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Abstract

Phloem-feeding insects are among the most devastating pests worldwide. They not only cause damage by feeding from the phloem, thereby depleting the plant from photo-assimilates, but also by vectoring viruses. Until now, the main way to prevent such problems is the frequent use of insecticides. Applying resistant varieties would be a more environmental friendly and sustainable solution. For this, resistant sources need to be identified first. Up to now there were no methods suitable for high throughput phenotyping of plant germplasm to identify sources of resistance towards phloem-feeding insects. In this paper we present a high throughput screening system to identify plants with an increased resistance against aphids. Its versatility is demonstrated using an *Arabidopsis thaliana* activation tag mutant line collection. This system consists of the green peach aphid *Myzus persicae* (Sulzer) and the circulative virus *Turnip yellows virus* (TuYV). In an initial screening, with one plant representing one mutant line, 13 virus-free mutant lines were identified by ELISA. Using seeds produced from these lines, the putative candidates were re-evaluated and characterized, resulting in nine lines with increased resistance towards the aphid. This *M. persicae*-TuYV screening system is an efficient, reliable and quick procedure to identify among thousands of mutated lines those resistant to aphids. In our study, nine mutant lines with increased resistance against the aphid were selected among 5160 mutant lines in just 5 months by one person. The system can be extended to other phloem-feeding insects and circulative viruses to identify insect resistant sources from several collections, including for example genebanks and artificially prepared mutant collections.

Key words

phloem-feeding insect, *Myzus persicae*, *Turnip yellows virus*, *Arabidopsis thaliana*, activation tag

Introduction

Phloem-feeding insects are among the most devastating pests worldwide, not only because of the direct damage caused by feeding, but also because of the viruses that many of them transmit. Viruses may be transmitted in a non-circulative or circulative way. In case of non-circulative viruses, like the potyviruses, the insect acquires the virus after a brief probe in an epidermal cell of a virus-infected plant. Subsequent probing on other (healthy) plants will transmit the virus from the aphids' stylet to the plants (Pirone and Blanc, 1996). Conversely, viruses that are transmitted in a circulative way, like members of the *Luteoviridae* family, are located in the phloem of the plant and insects can only acquire the virus by feeding for a prolonged period of time (up to 24 hours) from the phloem sap of infected plants (Hogenhout et al., 2008). The virus particles, taken up together with the phloem sap during feeding, cross the epithelial cells to diffuse through the haemolymph, and to finally be transported through the accessory salivary gland cells into the saliva and into a new plant during a subsequent feeding (Gildow, 1987). Once acquired, the virus can be maintained in the insect during the rest of its life. The efficiency of virus transmission is affected by plant traits conferring resistance against the vector insect. For instance, mechanical barriers may interfere with the insect's ability to reach the phloem and subsequently reduce the transmission of virions (Rodriguez-Lopez et al., 2011).

Most phloem-feeding insects are able to transmit more than a 100 different plant viruses (Hogenhout et al., 2008; Brault et al., 2010).Due to genomic variation and high mutation rate, it is relatively easy for plant viruses to overcome the resistance of plants (Tang and Leisner, 1997; Harrison, 2002). Therefore, it becomes an attractive strategy to search for resistance against the vector insect rather than for the resistance against each individual virus. At present, the main way to control phloem-feeding insects is via the frequent use of insecticides, which is only partly successful and hazardous to the environment. A more sustainable solution would be the use of plant varieties that are resistant to the insect. To be able to develop such resistant varieties, it is of utmost importance to identify resistant sources by screening plant collections, including genebank accessions or varieties, landraces and crop wild relatives, natural populations or even mutant collections (Agrawal, 2007; Broekgaarden et al., 2011; Lu et al., 2011; Kloth et al., 2012). In laboratory or green house experiments, plant resistance is normally quantified by using intact plants, detached leaves or even leaf disks to determine insect preference, population growth, survival and/or fecundity (Poch et al., 1998; Sharma et al., 2005; Müller-Schwarze, 2009; Pelletier et al., 2010; Maharijaya et al., 2011). In field experiments insect resistance is usually measured by monitoring natural infestation levels (Sharma et al., 2005). These commonly used techniques are very time consuming due to the need of regular observations and tedious counting. Therefore, only relatively small collections have been screened for insect resistance so far, which seriously reduces the chance of identifying new resistant sources.

Here, we present a method that allows the screening of large plant collections for resistance towards phloem-feeding insects, using a circulative virus as indicator. We demonstrate the versatility of the method by screening a collection of *Arabidopsis thaliana* mutant lines (Marsch-Martinez et al., 2002) for increased resistance towards the aphid *Myzus persicae* using the *Turnip yellows virus* (TuYV) as an indicator. These mutant lines harbour a randomly inserted transposon bearing the *Cauliflower mosaic virus* (CaMV) 35S promoter (Marsch-Martinez et al., 2002). Expression of genes located adjacent to the transposon may be increased leading to a gain-of-function phenotype (Marsch-Martinez et al., 2002). The different mutated lines were inoculated using viruliferous aphids and plants escaping infection were looked for. Because this virus does not show any symptoms on *A. thaliana*, we performed double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) to detect infected plants. This aphid-virus system enabled a single person to phenotype 5160 *A. thaliana* mutant lines in five months and to identify nine mutant lines with increased aphid resistance.

Materials and methods

Aphids, plants and virus

Myzus persicae (Verbeek et al., 2010) was reared in cages on Chinese cabbage (*Brassica rapa* L. ssp. *Pekinensis* cv. Granaat). The rearing was maintained in an environment controlled room with a relative humidity of 60-70%. The temperature was set to 20 ± 2 °C with an 18:6 L:D photoperiod. For all experiments, only apterous aphids were used.

A total of 5160 T-DNA activation-tag mutant lines of the *A. thaliana* accession Wassilewskija (WS) were obtained from the library present at Wageningen UR plant breeding (Marsch-Martinez et al., 2002). Plants were cultivated in a climate chamber, programmed for a 6:18 L: D photoperiod. The temperature was maintained at 20 $\underline{+2}$ ^{o}C during the day, 18 $\underline{+2}$ ^{o}C during the night. The relative humidity was kept at 60-70%. Plants were grown on rockwool and supplemented with Hyponex nutrition solution every two days (Tocquin et al., 2003). Three-week-old plants were used for all experiments. For seed collection plants were transferred, with the rockwool attached, into soil and placed in a greenhouse compartment at 20-22 ^{o}C with an 18:6 L:D photoperiod and a relative humidity of 60-70%.

*Turnip yellows virus (*TuYV; family *Luteoviridae*, genus *Polerovirus)* was kindly provided by Dr. Véronique Brault of INRA Colmar, France. The virus was maintained on *Physalis floridana* plants that were kept in a cage located in the same growth chamber as the *A. thaliana* plants.

Plant infestation/ virus transmission

Aphids were collected from Chinese cabbage and released on detached leaves of TuYV infected *Physalis* plants (Smyrnioudis et al., 2002) and allowed to feed for 48 hours (Gu, 1987) to obtain TuYV-viruliferous aphids. We used nymphs and adults to maximize the chances for successful TuYV transmission in our screening (Gill, 1970; Namba and Sylvester, 1981). Two first- and second-instar nymphs together with two other third- and fourth-instar nymphs were transferred onto each *A. thaliana* plant using a fine brush. At 5 dpi, aphids were eliminated by applying 2 ml per plant of systemic insecticide, Admire, (0.05 gram/l; Bayer Cropscience) onto the rockwool.

Virus detection by Double Antibody Sandwich-Enzyme linked immunosorbent assay

Because TuYV does not show any symptoms on *A. thaliana*, we conducted DAS-ELISA to detect the virus in plants. Two weeks post infestation with TuYV-viruliferous aphids two samples of newly developed leaves (approximately two square centimetres) were collected from each plant for the ELISA test. After leaf sample collection, plants were sprayed with BASTA (1 ml/li; Bayer Cropscience) to eliminate mutant lines without transposon insertion (Marsch-Martinez et al., 2002). Only data from plants surviving the BASTA treatment were taken into account for further analysis. Leaf samples were kept in tubes (Corning, product #4408), which were filled with two metal balls (Ø 2 mm) and 200 µl of extraction buffer (0.01 M Phosphate Buffered Saline, pH 7.4, containing 1 ml/l Tween 20, 20 g/l of polyvinyl pyrrolidone and 2 g/l ovalbumine, grade VI). Plant tissue was grinded by using Retsch (American Instrument Exchange, 3519N MILL) at a frequency of 30 cycles/second for one minute. One hundred µl plant extraction was analyzed by DAS-ELISA in immuno plates (Corning, product #9018) essentially as described by Clark and Adams in (Clark and Adams, 1977). Previous to the ELISA procedure plates were coated o/n at 4°C with 100 µl 1:1000 (v/v) dilution in coating buffer (1.59 gr Na2CO3, 2.94 gr NaHCO3, 0.5 gr NaN3, pH 9.6 /liter of coating antibodies against *Beet western yellows virus* (BWYV; the old name for TuYV). Antibodies were obtained from Prime Diagnotics (www.primediagnostics.com). Following incubation o/n at 4°c and washing plates

were incubated for 3 hours at 37°C with 100 µl 1:1000 (v/v) dilution of Alkaline phosphatase conjugated BWYV antibodies (www.primediagnostics.com). After a final wash, the immuno plates were incubated with substrate (0.75 mg paranitrophenylphosphate (pNPP) in 97 ml/l of diethanolamine, pH 9.8) at room temperature for half an hour. The absorbance value (A405 nm) was measured in Model 680 Microplate Reader (Bio-Rad Laboratories (UK)) (Bio-RAD Model 680XR). To establish a threshold value for healthy plants, each immuno plate also contained eight samples of non-inoculated *A. thaliana* wild type plants. The absorbance values of these healthy samples were used to calculate a threshold for each plate, which was the average healthy value plus three times their standard deviation. Plant samples with absorbance values higher than the threshold were considered positive for infection with the virus.

Aphid performance assay

To determine whether the candidate lines selected by the *M. persicae*-TuYV screening system were indeed aphid resistant mutant lines, we performed aphid assay in which the nymph pre-reproductive period and the population development on the candidate mutant lines were compared to those on wild type plants. Synchronized one-day-old nymphs were used to infest three-week-old *A. thaliana* plants with one nymph per plant. For the pre-reproductive period, the aphids were monitored twice a day at nine in the morning and at three in the afternoon from 6 till 12 dpi onwards. The time that a nymph began to reproduce was recorded. For the population development, the total number of aphids was counted at 14 dpi. After aphid number determination, plants in mutant lines were sprayed with BASTA to remove plants without transposon insertion. There was a minimum of 16 plants for each candidate mutant line as well as for wild type plants. Comparisons for aphid performance between mutant lines and wild type were analyzed by independent-samples t-tests. $p<0.05$ was used to detect statistical differences.

Results

Selection and re-evaluation of aphid resistant candidates by the M. persicae-TuYV system

A total of 5160 mutant lines of *A. thaliana* were evaluated in four batches. Four viruliferous aphids were released on each plant for virus transmission and one plant per mutant line was tested. Leaf samples from 1280 mutant lines in the first batch were examined for TuYV infection by ELISA at 14 and 21 days post infestation (dpi) as TuYV does not show any symptoms on *A. thaliana*. This revealed that 99.9% of the mutant lines were infected at 14 dpi, i.e. one mutant line (4619) showed negative ELISA values whereas all others were positive, and 100% of them were infected at 21 dpi. To increase the chances of finding candidate mutants that may express partial increased resistance to aphids, the remainder of the mutant lines were tested at 14 dpi and 13 mutant lines were negative when assayed by ELISA result, indicating no or a very low virus concentration. To confirm the absence of virus infection of the 13 mutant lines, seeds were generated from these lines by selfing and 30 plants per mutant line were re-evaluated using the *M. persicae*-TuYV system. For nine mutant lines, a fraction of the plants showed a negative ELISA result, indicating that the virus was absent. Per mutant line tested the percentage of non-infected plants varied from 3.3% to 20% depending of the mutant line (Table 1). The remaining four mutant lines behaved like the wild type plants showing 100% of infection (Table 1).

Table 1. Frequency of non-infected plants and infection level in mutant lines and wild type

Mutant lines	Frequency of non-infected plants	Number of plants analyzed	Mean OD ± SD
Wild type plants	0	30	0.44± 0.13
807	0	22	0.41± 0.12
1912	0	30	0.37± 0.14
402	0	27	0.33± 0.12
1264	0	25	0.38± 0.14
1348	3.3	30	0.40± 0.14
3537	3.3	30	0.56± 0.16
3646	4.8	21	0.51± 0.12
3732	6.7	30	0.36± 0.15
2018	8.7	23	0.39± 0.09
3790	10	30	0.30± 0.12
4619	17	30	0.49± 0.21
3474	17	30	0.34± 0.11
1378	20	30	0.37± 0.15

Seeds were generated from selfed candidate mutant lines. Around thirty plants per mutant line were re-evaluated in *M. persicae*-TuYV system as described in "Methods". ELISA values were means optical density (OD) ± standard deviation (SD) of infected plants, with 0.073±0.003 for non-inoculated plants.

Characterization of the candidate mutant lines by aphid assays

The absence or the low viral infection of the selected mutated lines can be explained by a resistance of the plant to the virus or to the aphid. In order to discriminate between these two possibilities, aphid performance on the candidate line was followed. We monitored the pre-reproductive period and the population development of synchronized one day old nymphs. Aphid behavior was negatively affected on the nine mutant lines for which a certain percentage of virus free plants were found in the re-evaluation of the aphid-virus system (Figure 1). Six mutant lines showed a delayed time to reproduction compared to the wild type, ranging from 0.5 to 1 day (Figure 1A). Aphid population size 14 dpi was significantly lower, up to 40% less, on all these nine mutant lines compared to the wild type (Figure 1B).

We also included in our analysis the four mutant lines that were initially identified by the aphid-virus system as candidates, but showed to be false negatives after re-assessment of the progeny, as all the plants of these mutant lines were infected by the virus in the re-evaluation. On two mutant lines (807 and 1912) the nymphs began to reproduce one day earlier than the nymphs on the wild type plants (Figure 1A). Subsequently, those two lines contained significantly more aphids than the wild type plants at 14 dpi (Figure 1B). For mutant line 1264 both the nymph development and the population development were comparable to that of the wild type plants; whereas the time to reproduce on mutant line 402 was slightly delayed but aphid population reached the same level as the one on wild type plants at 14 dpi (Figure 1).

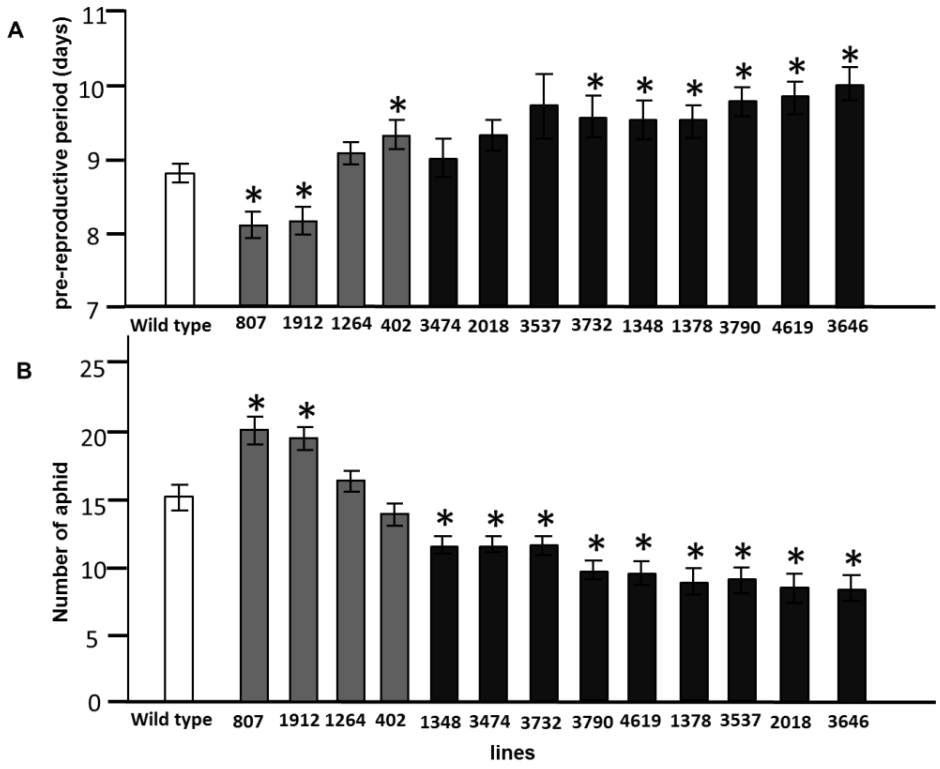

Figure 1 Aphid performance on mutant lines and wild type. Synchronized one-day-old nymphs were used to infest three-week-old *A. thaliana* plants with one nymph per plant. The time that a nymph began to reproduce was recorded (A). The total number of aphids was counted at 14 dpi (B) Values are the means ± SE of at least 16 plants. The asterisks indicate a significant difference compared to the wild type plants ($p < 0.05$, Independent-samples *t*-test).

Discussion

The aphid-virus system

In this paper we present an aphid-virus system that allows the screening for aphid resistance of a large collection of plants. Its versatility is demonstrated using an *A. thaliana* activation tag mutant collection (Marsch-Martinez et al., 2002). In total 5160 mutant lines were tested using this system -by one person in five months-, resulting in the identification of nine mutant lines showing an increased level of resistance towards aphids. Previously, another *A. thaliana* activation tag mutant collection has been phenotyped for altered glucosinolate content after which the candidate lines were evaluated for resistance towards *M. persicae* (Weigel et al., 2000; Levy et al., 2005). This resulted in the identification of only one aphid resistant mutant line (*IQD*1) out of 16500 (Weigel et al., 2000; Levy et al., 2005). This targeted approach, i.e. selecting candidate lines based on altered glucosinolate content and then characterizing the lines with increased levels of glucosinolates for insect resistance, may explain the relative low number of insect resistant mutant lines identified in that study. To our knowledge, this aphid-virus system is the first method adapted to screen large collections of plants for resistance to phloem-feeding insects in an untargeted way. Using this approach many genes affecting the level of aphid resistance can be identified. The success in narrowing down the number of putative candidates was attributed to the use of TuYV, a circulative virus that can only be efficiently transmitted during phloem ingestion by the aphid. Certain plant traits may affect the aphid's feeding behavior and consequently the possibility and efficiency of virus transmission into plants. For instance, probing capability of the whitefly *Bemisia tabaci* has been shown to be reduced on tomato

plants with acylsucrose-secreting type IV trichomes that consequently reduced the spread of *Tomato yellow leaf curl virus* (Rodriguez-Lopez et al., 2011).

The *M. persicae*-TuYV system can be easily used to screen large collections of plants in comparison to other time consuming and labour intensive methods that are used for identifying aphid resistant sources. For example, the arena setup is a frequently used method in which aphids are released in the middle of a circle formed by different plants and are allowed to choose a plant to feed on for a certain time period after which the number of aphids on each plant is counted (Poch et al., 1998). Another commonly used method is based on non-choice tests in which aphids are confined to a plant or a specific leaf area by insect-proof cages and let to produce offspring (Pelletier et al., 2010; Firdaus et al., 2011). For all these methods, regular monitoring and counting of aphid numbers is required to compare the insect preference/performance between plants which limits their applicability for screening large collections. When using the *M. persicae*-TuYV system thousands of plants can be grown at one time and tedious counting work is not required. The screening system holds the middle between a choice and non-choice assay, i.e. aphids and nymphs are transferred directly onto each plant, but the aphids/nymphs can move freely to other plants. This means that attraction/repellence, which can be influenced by the virus (Alvarez et al., 2007; Mauck et al., 2010), may affect the outcome of the assay and increase or decrease the number of resistant candidates.

Plant traits that negatively affect aphid feeding behavior may affect the timing of virus transmission and/or the number of virions that will be transferred into the phloem. Therefore, we hypothesized that mutant plants expressing aphid resistance traits would have a lower chance of getting infected, are infected at a later stage or are infected with fewer virus particles resulting in a longer time for the virus to develop into a detectable level than for wild type plants. When 30 individuals of each candidate mutant line with increased aphid resistance were tested, nine mutant lines were considered true candidates because they showed partially negative results in the ELISA test, ranging from 3.3 to 20 % of non-infected plants (Table 1). Detection of the virus in plants from mutant lines with increased aphid resistance in the re-evaluation can be explained by this hypothesis as the percentage of infected plants was lower for these lines compared to the wild type indicating a lower chance of getting infected for the mutant lines. That this hypothesis is likely correct was shown for mutant line 4619 that was tested as not infected at 14 dpi but found infected at 21 dpi.

It should be noted that the high throughput trades off with a relative high chance of overlooking candidates with increased resistance to aphids. Applying a shorter time for virus development, e.g. 7 dpi, may increase the number of aphids resistant candidates, but it may come with the disadvantage of more false candidates as well.

As our method included one plant per mutant line only, there is a risk of missing aphid resistant candidates. The heterogeneity of some mutant lines, revealed by the absence of the BASTA resistance gene (the selectable marker present on the transposon), may have resulted in overlooking some candidate mutant lines. Obviously, this limitation can be overcome by testing more plants per mutant line. Additionally, when the nine confirmed mutant lines with increased resistance to aphids were re-evaluated with more individuals using the aphid-virus system, they produced on average 10% non-infected plants (table 1), suggesting that more aphid resistant mutant lines are present in the activation tag mutant collection that have not been identified in the initial screening.

Mutant lines selected

All candidate mutant lines showed a reduced population development with non- viruliferous aphids (Figure 1B), indicating that plants with partial resistance to the aphid can be selected using our

method. *Arabidopsis thaliana* is a suitable host to *M. persicae* and to our knowledge no accessions or mutant lines expressing a complete resistance to this aphid have been reported. Available literature shows differences in susceptibility levels only (Mewis et al., 2005; Mewis I, 2006.; Louis et al., 2010), which are comparable to the differences in population development between our mutant lines and the wild type. Due to the susceptibility of *A. thaliana*, it is not likely to identify *A. thaliana* mutant lines with full resistance against aphids (Smith and Boyko, 2007), which is confirmed in our study. Part of this reduction in aphid population development may be explained by a longer pre-reproductive period, but this is not the case for mutant lines 3474, 2018 and 3537. On these three lines, nymphs developed into adults similarly as on the wild type plants, suggesting that the increased resistance of the plant mainly affected the fecundity of the aphids.

Surprisingly, on two mutant lines that were initially selected but were found to be false negatives in the confirmation screen aphids showed a shorter pre-reproductive period and a larger population size than on wild type plants (Figure 1), meaning these two lines are better hosts to the aphids than wild type plants. This was completely contradictory to our expectations since this system was expected to identify mutant lines with a reduced aphid performance. So far we do not have any explanation for this unexpected finding.

Aphids have been widely used to study virus transmission and the mechanisms of plant resistance to virus (Whitham et al., 1999; Smyrnioudis et al., 2002). However, it has been reported that the identified plant resistance to virus may actually be due to resistance against the vector aphids. For example, resistance to *Barley yellow dwarf virus* in some *Arogyron* species was due to the inability of aphid to reach the phloem [30]. In our screening we did not find any virus resistant mutant lines, which probably means that more lines need to be tested to identify such resistance.

Application

We have provided proof-of-concept for the versatility of the aphid-virus system using an *A. thaliana* activation tag mutant collection and the aphid *M. persicae*. Since *M. persicae* is not the only phloem-feeding insect that can vector plant viruses, our system can also be transposed to other phloem-feeding insects and circulative viruses as well as to other plant collections, i.e. other mutant libraries or genebank collections containing crops or crop wild relatives. For instance, the system may be used to identify plants with increased resistance to the whitefly *B. tabaci* using a geminivirus or the *Lettuce infectious yellows virus* as an indicator (Bedford et al., 1994; Tian et al., 1999). Similarly, resistance to corn planthopper *Graminella nigrifrons* and *Peregrinus maidis* may be identified with *Maize chlorotic dwarf virus* and *Sorghum stripe virus* as indicator respectively (Choudhury and Rosenkranz, 1983; Narayana and Muniyappa, 1996). In addition to plant viruses, phytoplasmas are mainly transmitted by leafhoppers and psyllids that are also phloem-feeding insects (Ploaie, 1981). Similar to the circulative plant virus, the phytoplasmas are taken up by the insect during phloem ingestion on an infected plant, cross the insect gut, amplify in the hemolymph, and circulate into the salivary glands. Then, the insect transfers the phytoplasmas to any plant when feeding (Agrios, 1997). Therefore, our insect-virus system could be applied in such combination for which circulative phytoplasmas may serve as an indicator for plant resistance against leafhoppers and psyllids.

We had used ELISA to detect the virus as it does not show any symptoms on *A. thaliana*. However, in a lot of cases one can use the virus symptoms as an indicator and thus circumvent the ELISA test. For instance, *Cucumber mosaic virus* infected tomato shows the deformation of leaves with stunted growth (Murphy et al., 2000); *Tomato yellow leaf curl virus* causes clear yellowing and curling symptoms on plant leaves (Picó et al., 1996), and *Potato virus Y* causes necrosis on potato leaves (Kassanis and Nixon, 1961). When a virus does not show any symptoms one may also consider developing an

engineered virus that will induce symptoms development, or adding the gene for the production of green fluorescent protein (GFP) (Tsien, 1998) to the virus to visualize the presence of the virus in the plant. When a virus shows an asymptomatic infection or when symptoms can be induced by nutrient deficiencies (Uchida, 2000) then molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) can be used to detect the virus (Huhnlein et al., 2010).

Conclusion

In this paper we present a high-throughput phenotyping system, in which TuYV serves as an indicator for *M. persicae* resistance in *A. thaliana* plants. This aphid-virus system is a reliable method to identify candidates with increased resistance in a large plant collection. During the screening of 5160 mutant lines, nine lines with increased aphid resistance were identified. The aphid-virus system may be developed for other insect-virus combinations.

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Chapter 4

Overexpression of *IRM1* enhances resistance to aphids in *Arabidopsis thaliana*

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Abstract

Aphids are insects that cause direct damage to crops by the removal of phloem sap, but more importantly they spread devastating viruses. Aphids use their sophisticated mouthpart (i.e. stylet) to feed from the phloem sieve elements of the host plant. To identify genes that affect host plant resistance to aphids, we previously screened an *Arabidopsis thaliana* activation tag mutant collection. In such mutants, tagged genes are overexpressed by a strong 35S enhancer adjacent to the natural promoter, resulting in a dominant gain-of-function phenotype. We previously identified several of these mutants on which the aphid *Myzus persicae* showed a reduced population development compared with wild type. In the present study we show that the gene responsible for the phenotype of one of the mutants is At5g65040 and named this gene *Increased Resistance to Myzus persicae 1* (*IRM1*). Overexpression of the cloned *IRM1* gene conferred a phenotype identical to that of the original mutant. Conversely, an *IRM1* knockout mutant promoted aphid population development compared to the wild type. We performed Electrical Penetration Graph analysis to investigate how probing and feeding behavior of aphids was affected on plants that either overexpressed *IRM1* or contained a knockout mutation in this gene. The EPG results indicated that the aphids encounter resistance factors while reaching for the phloem on the overexpressing line. This resistance mechanism also affected other aphid species and is suggested to be of mechanical nature. Interestingly, genetic variation for *IRM1* expression in response to aphid attack was observed. Upon aphid attack the expression of *IRM1* was initially (after 6 hours) induced in ecotype Wassilewskija followed by suppression. In Columbia-0, *IRM1* expression was already suppressed six hours after the start of the infestation. The resistance conferred by the overexpression of *IRM1* in *A. thaliana* trades off with plant growth.

Key words

phloem-feeding insect, *Myzus persicae*, *Brevicoryne brassicae*, activation tag mutant, electrical penetration graph (EPG), constitutive overexpression

Introduction

Phloem-feeding insects display a variety of activities during settlement and feeding on a host plant. Aphids, for example, choose a plant based on surface cues (Walling, 2008). After landing on a host plant, they intercellularly probe plant tissue and frequently puncture epidermis, mesophyll, and parenchyma cells to determine the suitability of the host (Tjallingii, 1985). Once they established a feeding site, aphids can continue feeding from a single phloem sieve element for hours or even days (Halarewicz and Gabryś, 2012). These probing and feeding activities of aphids can be monitored using the Electrical Penetration Graph (EPG) technique in which the aphid and the plant are wired in a low-voltage circuit connected to a recording system (Tjallingii, 1990; Tjallingii et al., 2010). Information on the aphid activities can be extracted from the recorded signal waveforms and provides insight into the location of plant resistance factors (Tjallingii et al., 2010).

Plants are not passive victims of insect attack but they have developed several lines of defense (Broekgaarden et al., 2011). Plant defenses can be based on chemical and/or mechanical traits that negatively affect the biology of the insect (Will and van Bel, 2006). Chemical defense usually involves compounds with antibiotic activity that are present on the leaf surface or in the phloem (Wagner et al., 2004; Firdaus et al., 2011). For instance, secondary metabolites present in trichomes of tomato prevent aphids from settling (Simmons et al., 2005). Similarly, a protein possessing lectin activity in *Arabidopsis thaliana* has an insecticidal effect towards aphids (Vasconcelos and Oliveira, 2004; Beneteau et al., 2010). Structural modifications of the cell wall may hamper aphid feeding by strengthening barriers against probing and feeding. Transcript profiling studies revealed that genes encoding proteins associated with cell wall reinforcement and remodelling were commonly up-regulated in aphid infested plants (Thompson and Goggin, 2006; Divol et al., 2007; Kusnierczyk et al., 2008).

Some genes may potentially affect resistance towards aphids once their expression level or profile is changed (Levy et al., 2005; Zhang et al., 2006). For the identification of such genes activation tag mutant libraries can be used. In an activation tag mutant, genes are overexpressed to generate a dominant gain-of-function phenotype that can be selected for (Levy et al., 2005). The activation of genes is accomplished by random insertion of a transposon on which the *Cauliflower mosaic virus* (CaMV) 35S promoter is present that can constitutively enhance or activate the expression of adjacent genes (Marsch-Martinez et al., 2002). Previously, we used this *A. thaliana* activation tagged population to screen for resistance towards the aphid *Myzus persicae* with the aid of an aphid-virus system in which the efficiency of virus transmission was used as an indicator for aphid resistance. This screen resulted in the identification of nine mutants with and increased resistance towards *M. persicae*, i.e. slower aphid population development on the mutant compared to the wild type (Chen et al., 2012). In this paper, we describe the characterization of one of these mutants by identifying the activated gene and its role in aphid resistance. This led to the identification of the *Increased Resistance to Myzus persicae 1* (*IRM1*) gene that, once being overexpressed, increased the resistance of *A. thaliana* towards aphids.

Materials and methods

Insect rearing

Myzus persicae was reared in cages on Chinese cabbage (*Brassica rapa* L. ssp. *Pekinensis* cv. Granaat). *Brevicoryne brassicae* was reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus) at the Department of Entomology, Wageningen University. Both rearings were maintained

in an acclimatized room with a relative humidity of 60-70%, a temperature of 20 ± 2 ºC and an 18:6 L:D photoperiod. For all experiments, only apterous aphids were used.

Plant material and growth conditions

Mutant 3646 was previously identified as a mutant with a reduced aphid population development (Chen et al., 2012). Seeds of *A. thaliana* wild type Wassilewskija (WS) were obtained from the library present at Wageningen UR Plant Breeding (Marsch-Martinez et al., 2002). Seeds of T-DNA insertion line SALK_106042 (At5g65040 knock out mutant, referred to as 40-KO hereafter and its corresponding wild type Columbia-0 (Col-0) were obtained from NASC (http://arabidopsis.info/; (Scholl et al., 2000)). To induce germination, seeds were placed at 4 ºC in the dark for 3 days under high humidity. Subsequently, seeds were transferred to potting compost (Lentse Potgrond®) and plants were cultivated in a climate chamber with a 6:18 L:D photoperiod. The temperature was maintained at 20 ± 2 ºC during the day and 18 ± 2 ºC during the night (60-70% relative humidity). Plants were watered every other day and no pest control was applied.

Confirmation of homozygous presence of T-DNA in the 40-KO mutant

Genomic DNA of 40-KO leaves was isolated using the DNeasy Plant Mini kit (Qiagen). A PCR reaction was carried out to confirm the homozygous presence of the T-DNA insertion in the first exon of the At5g65040 gene (Supplemental Figure 1). Gene specific primers 40-KO_F and 40-KO_R) were designed up- and downstream of the T-DNA insertion site (http://signal.salk.edu/tdnaprimers.2.html) and used in combination with a T-DNA left border primer (LBb1.3) (Table 1). PCR reactions were performed in a total volume of 20 µl according to the manual of Phire® (Finnzymes, Product codes: F-122S). The PCR programme consisted of 30 seconds at 98 ºC followed by 35 cycles of 98 ºC for 5 sec, 63 ºC for 5 sec, and 72 ºC for 30 sec with a final extension at 72 ºC for 10 min.

Table 1. Primer sequences.

name	purpose	sequence (5'--3')
Bar_F	Inverse PCR	GCGTCGTTCTGGGCTCATGGT
Bar_R	Inverse PCR	CTGGCAGCTGGACTTCAGCCTG
T-DNA LB_F	Inverse PCR	CCCGTCTCACTGGTGAAAAGAA
T-DNA LB_R	Inverse PCR	ATTCGGCTATGACTGGGCACA
LBb1.3	Confirmation of T-DNA insertion	ATTTTGCCGATTTCGGAAC
40-KO_F	Confirmation of T-DNA insertion	CACGAACAAATCAAATCATGC
40-KO_R	Confirmation of T-DNA insertion	TGAAAATTTGAATTCACTGGTTG
At5g65040_F	Quantitative RT-PCR	TCTGCCATCATCGTGACATT
At5g65040_R	Quantitative RT-PCR	TTTGCTTCTCCCTGCATTCT
At5g65050_F	Quantitative RT-PCR	GGAATGTCATGGGAAAATGG
At5g65050_R	Quantitative RT-PCR	AGCTCAGCCGTTGATGATG
Actin8_F	Quantitative RT-PCR	GATGGAGACCTCGAAAACCA
Actin8_R	Quantitative RT-PCR	AAAAGGACTTCTGGGCACCT
AttB1F	Construction of transgenic plant	GGGGACAAGTTTGTACAAAAAAGCAGGCT
AttB2R	Construction of transgenic plant	ACCACTTTGTACAAGAAAGCTGGGT

Construction of transgenic *A. thaliana* plants

The full length coding region of At5g65040 attached to a forward primer AttB1F (located upstream of the start codon) and reverse primer AttB2R (located downstream of the stop codon) situated in the pEX-A vector was obtained from Eurofins (Ebersberg, Germany). The coding region fragment of At5g65040 was transferred into donor vector pDONR207 using the Gateway® BP Clonase™ II enzyme mix (Invitrogen) to generate entry vector pDONR207::At5g65040. The entry vector was subsequently cloned into Gateway destination vector pFAST-R02 (Shimada et al., 2010) using the Gateway LR® Clonase™ II enzyme mix (Invitrogen) to generate the expression construct pFAST-R02-40 in which At5g65040 is under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. The construct was transformed into *E. coli* and transformants were checked by colony PCR analysis using primers AttB1F and AttB2R (Table 1). After verifying the accuracy of the sequences of the gene, the construct was transformed into *Agrobacterium tumefaciens* strain GV3101 (Hellens et al., 2000) by electroporation. *Agrobacterium tumefaciens* mediated transformation (Clough and Bent, 1998) was used to introduce the pFAST-R02-40 plasmid into Columbia-0 and 40-KO mutant plants. Seeds containing the construct were selected using fluorescence microscopy (Zeiss, SteREO Discovery.V8) (Shimada et al., 2010). Two independent transformants in Col-0, referred to as G0085 and G0088,and two independent transformants in 40-KO, referred to as G0090 and G0092 , were used in further experiments.

Inverse PCR

Genomic DNA of leaves collected from mutant 3646 was extracted using the DNeasy Plant Mini kit (Qiagen). Isolated DNA was digested with restriction enzyme EcoRI (Thermo, product # ER0275) or BamHI (Thermo, product # ER0051) and subsequently ligated with T4 DNA ligase (Fermentas, product # EL0011). Five µl of ligated DNA was used as a template in an inverse PCR (iPCR) reaction that was performed in a total volume of 50 µl containing the Phusion™ enzyme (Finnzymes, Product codes: F-530S, 100U). All enzymes were used according to the supplier's manuals. Primers were designed with Primer-3-Plus (Untergasser et al., 2007). For transposon flanking sequence isolation, primers Bar_R and Bar_F were designed based on the sequences of the *BAR* gene that is located on the transposon (Table 1). For T-DNA flanking sequence isolation, primers (T-DNA LB_F and T-DNA LB_R) were designed based on the sequences of the T-DNA left border (Table 1), since the right border of T-DNA is commonly lost upon integration (Weigel et al., 2000). The following iPCR programme was used: 30 seconds at 98 ºC followed by 35 cycles of 98 ºC for 10 sec, 64 ºC for 10 sec, and 72 ºC for 3 min with a final extension at 72 ºC for 10 min. PCR products were sequenced and then blasted against the *A. thaliana* genome (http://www.arabidopsis.org/; (Altschul et al., 1990)).

Time course experiment of aphid infestation

Four-week-old wild type plants were infested with 15 randomly selected aphids per plant. Plant material was collected at zero, six and 24 hours after aphid infestation. Aphids were gently brushed away from the leaf tissue. Uninfested *A. thaliana* plants were also brushed. For each treatment, three biological replicates were obtained each consisting of a pool of 17 plants. Leaf samples were immediately flash frozen in liquid nitrogen after collection and stored at -80 °C until use.

Quantitative RT-PCR

Total RNA from leaf samples was extracted using the RNeasy Plant mini kit (Qiagen). One µg of total RNA was treated with DNaseI (Invitrogen) according to the manufacturer's instructions. DNA-free total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). Synthesised cDNA was diluted 20 times. Gene-specific primers were designed with Primer-3-Plus

software (Untergasser et al., 2007) and are listed in Table 1. The *ACTIN8* (At1g49240) gene was used as the reference to normalize gene expression across the samples (Iven et al., 2012). Quantitative RT-PCR was performed in a total volume of 10 µl containing 2 µl cDNA, 1.5 µl of each gene-specific primer (0.5µM), and 5 µl SYBR Green Supermix Reagent (BioRad). Quantitative RT-PCR was performed in duplicate in a Real-Time Thermal Cycler (BioRad) using the following programme: 95 ºC for 3 min followed by 40 cycles of 95 ºC for 15 sec, and 60 ºC for 1 min.

No-choice aphid assays

No-choice aphid assays were performed with 15 biological replicates per genotype. Synchronized one-day-old nymphs were used to infest three-week-old plants with one nymph per plant. Nymphs were transferred to the plants using a fine brush. The total number of aphids was counted 14 days after infestation. Independent-samples *t*-test and ANOVA followed by Tukey tests were used to determine the significance between genotypes ($P< 0.05$).

Electrical penetration graph

The electrical penetration graph (EPG) technique (Tjallingii, 1990) was employed to monitor penetrating and feeding behavior of aphids on mutant and wild type plants. A gold wire (diameter 20 µm) was attached onto the dorsum of young adult aphids using conductive water-based silver glue. The wired aphid was placed on a five-week-old plant that was connected to a recording system via a copper electrode in the soil (Tjallingii, 2006). The EPGs were recorded in a 22 ºC room with constant light for 8 hours. At least 15 recordings of individual aphids (one aphid per plant) were obtained for each line. The EPG data were analyzed using the PROBE 3.0 software (Wageningen University, the Netherlands) to distinguish the various waveforms. Waveform C represents the pathway phase, when the aphid stylet is penetrating through the leaf tissue; waveform E2 represents phloem sap ingestion; Waveform F is associated with derailed stylet mechanics or penetration difficulties; and waveform G indicates active uptake of water from the xylem elements (Tjallingii, 1990).

Parameters were analyzed individually for each aphid after which the means and standard errors of the mean (SE) for the total number of aphids per genotype was calculated. The Mann-Whitney U and Fisher exact test were used to determine if there were significant differences in the aphid's probing and feeding behavior between mutant and wild type plants ($P< 0.05$).

Results

Phenotypic characterization of mutant 3646 and location of the T-DNA

Mutant 3646 was previously identified as an *A. thaliana* activation tag mutant with a significantly smaller number of *M. persicae* than the wild type WS (Chen et al., 2012). In addition, aphids showed a longer pre-reproductive period on this mutant compared to the wild type WS (Chen et al., 2012). Plants of mutant 3646 are dark green with smaller rosette leaves than the wild type (Figure 1A). Furthermore, mutant 3646 needed a longer time to flower, and had smaller flowers and siliques than wild type WS plants.

In an activation tag mutant, a mutation may be caused by either the transposon and/or the T-DNA on which the transposon is present (Marsch-Martinez et al., 2002). To determine the cause of the phenotype of mutant 3646, we performed inverse PCR with primers designed on transposon and T-DNA sequences (Table 1). The PCR fragments obtained with primers that amplify transposon flanking sequences were 100% identical to T-DNA sequences, indicating that the transposon was still located on the T-DNA. Primers designed to pick up T-DNA flanking sequences recovered *A. thaliana*

genomic DNA. Using BLASTn (Altschul et al., 1990), we determined that the T-DNA was located 3.5 kb upstream of gene At5g65040 and 0.5 kb upstream of gene At5g65050 (Figure 1B). Because the enhancer can effectively activate genes within a range of 8.2 kb (Ichikawa et al., 2003), these two genes were considered candidate genes responsible for the increased aphid resistance of mutant 3646.

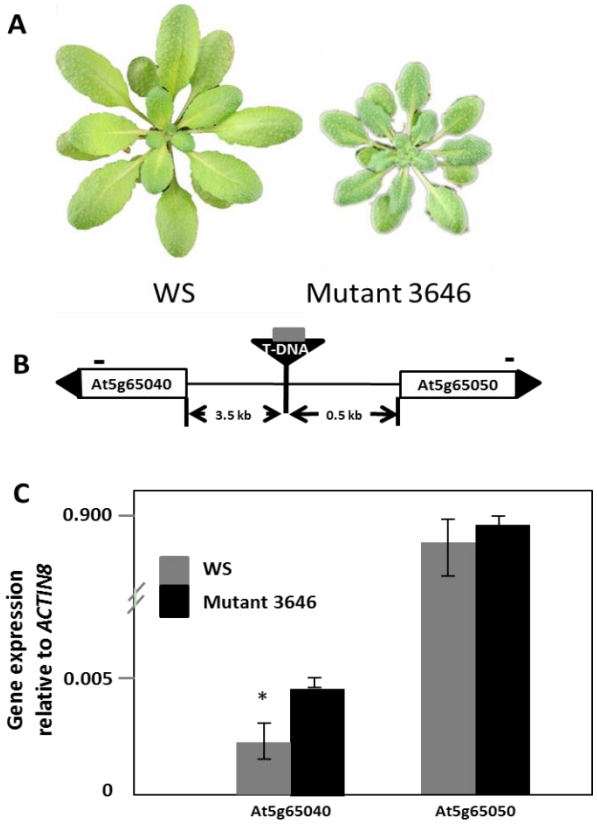

Figure 1. Characteristics of mutant 3646. (A) Phenotype of six week old Wassilewskija (WS) and activation tag mutant 3646; (B) Location of the T-DNA insert (inverted triangle) containing the transposon (grey square) between At5g65040 (*IRM1*) and At5g65050. Black triangles at the end of the genes indicate the gene orientation. The distance from a gene to the T-DNA is indicated below the horizontal line. Short lines above the genes represent the position of primers used for quantitative RT-PCR analysis. Diagram is not drawn to scale; (C) Quantitative RT-PCR analysis of the two genes flanking the T-DNA. Values are the means ± standard deviation of three biological replicates. The star indicates a significant difference between bars within a pair (Independent-samples *t*-test, $P < 0.05$).

Identification and verification of the gene responsible for the increased aphid resistance

To determine the responsible gene for the increased aphid resistance, we first performed quantitative RT-PCR to compare the expression level of the two candidate genes in mutant 3646 and wild type plants. Quantitative RT-PCR demonstrated a significantly higher expression of At5g65040 in mutant 3646 than in the wild type, whereas the expression of At5g65050 in mutant 3646 was at the same level as in wild type (Figure 1C). Therefore, At5g65040 was considered the prime candidate for the increased aphid resistance in mutant 3646.

To further verify the role of At5g65040 in resistance against *M. persicae* in *A. thaliana*, no-choice aphid assays were performed to compare aphid population development on At5g65040 knock out mutant plants (referred to as 40-KO hereafter) to that on plants of its corresponding wild type Col-0. The 40-KO mutant is morphologically similar to the wild type (Figure 2A) and it contains a T-DNA insert in the first exon of At5g65040 that disrupts the expression of this gene (Figure 2B, Supplemental Figure 1). Fourteen days after infestation, aphid numbers were significantly higher on 40-KO than on wild type Col-0 plants (Figure 2C). In addition, we constructed transgenic lines by overexpressing At5g65040 under the CaMV 35S promoter in wild type Col-0 (G0085, G0088) and 40-KO mutant (G0090, G0092) plants. Compared to the wild type, all the transgenic lines had smaller rosette leaves (Figure 2A), delayed bolting time and smaller size of flowers and siliques. The expression of At5g65040 was significantly higher in the transgenic lines than in the wild type Col-0 and the expression levels differed among the lines (Figure 2B). The numbers of aphids on these lines

were significantly lower than on the wild type (Figure 2C) 14 days after infestation. Taken together, these results confirm that At5g65040 is the gene responsible for the increased aphid resistance in mutant 3646 and we named this gene *Increased Resistance to Myzus persicae 1* (*IRM1*). To reveal how *IRM1* is expressed in wild type plants in response to aphid attack, we performed a time course experiment of aphid infestation. Figure 3A shows a significant induction of *IRM1* expression in WS, six hours after infestation followed by a repression later. In Col-0 the expression of *IRM1* was already repressed after 6 hours of aphid infestation and remained as such (Figure 3B).

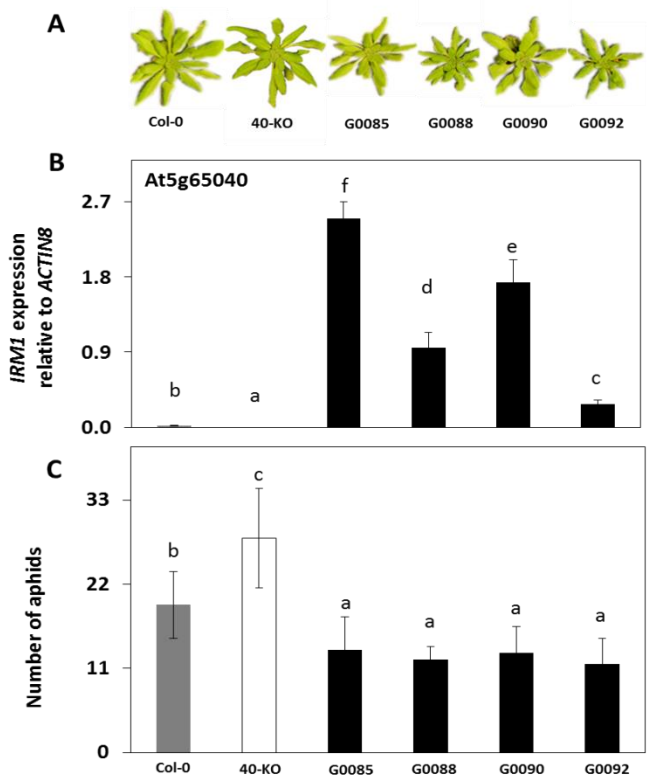

Figure 2. Characteristics of *IRM1* mutant lines and the effect of this gene on aphid performance. Phenotype of six week old Columbia-0 (Col-0), *IRM1* knock-out mutant (40-KO) and *IRM1* overexpressing transgenic lines (G0085, G0088, G0090, G0092); (B) Expression of *IRM1* in leaves of Col-0, *IRM1* knock out mutant and four independent *IRM1* overexpressing transgenic lines. Values are the means (± SD) of three biological replicates; (C) No-choice aphid assays on plants of Col-0, 40-KO and transgenic overexpressing lines. Values are the means (± SD) of 15 biological replicates. Bars marked with different letters are significantly different from each other (ANOVA followed by Tukey tests, $P < 0.05$).

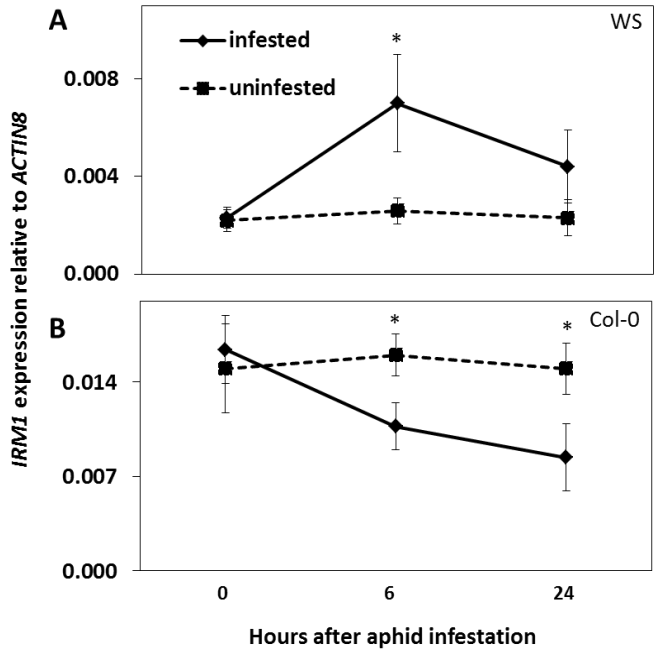

Figure 3. Expression analysis of *IRM1* in WS and Col-0 upon aphid infestation. Expression levels of *IRM1* in WS (A) and Col-0 (B) plants 0, 6 and 24 hours after aphid infestation. Values are the means (± SD) of three biological replicates. The stars indicate significance between infested and uninfested samples within a time point (Independent sample *t*-test, $P < 0.05$).

Aphid probing and feeding behavior on lines affected in *IRM1* expression

To obtain information about the possible role of *IRM1* in aphid resistance we recorded in detail the probing and feeding behavior of aphids on mutant (3646 and 40-KO) and wild type (WS and Col-0) plants using the EPG technique. All aphids started to penetrate the leaf they were placed on around the same time on all tested plants, as indicated by the time to the first probe (Table 2). The EPG parameters related to stylet pathway behavior showed significant differences between mutant 3646 and wild type WS (Mann-Whitney U test, d.f. = 33, $P < 0.05$; Table 2). A significantly larger number of test probes and a significantly longer time of the minimum of waveform C prior to sieve element salivation (E1) were observed on mutant 3646. Waveform F, associated with derailed stylet penetration, was also observed for a significantly longer time and in a larger number on mutant 3646 (Table 2). Significant differences were also observed for the pathway phase between mutant 40-KO and wild type Col-0 (Mann-Whitney U test, d.f. = 31, $P < 0.05$; Table 2), which was the opposite of the difference between mutant 3646 and wild type WS. On mutant 40-KO, the number of test probes was significantly smaller and minimum waveform C prior to sieve element salivation (E1) was shorter (Table 2). With regard to phloem-contact, parameters differed only between mutant 3646 and wild type WS. On mutant 3646 aphids needed more time from the first probe to the first sieve element salivation (1st E1) (Mann-Whitney U test, d.f. = 33, $P < 0.05$; Table 2) and to the first sustained phloem sap ingestion (1st sE2) (Mann-Whitney U test, d.f. = 28, $P < 0.05$; Table 2). Furthermore, a significantly smaller number of aphids on mutant 3646 reached the sustained phloem sap ingestion (sE2) during the eight hours recording (Fisher's exact test, two-tailed, $P < 0.05$; Table 2). For phloem feeding, however, aphids did not perform differently as indicated by comparable phloem sap ingestion (E2) between mutant and wild type plants (Table 2). In the xylem phase, a difference was observed only between mutant 40-KO and wild type Col-0 (Mann-Whitney U test, d.f. = 31, $P < 0.05$; Table 2) . The aphids spent less time taking up xylem sap from mutant 40-KO as was indicated by a shorter time and smaller number of waveform G (Table 2).

Table 2. Electrical penetration graph (EPG) results.

location of resistance factor	parameters and number		unit	WS	3646	*P* value	Col-0	40-KO	*P* value
	EPGs		#	18	15		16	15	
surface	time to 1st probe	1	min	2.5 ± 0.7	3.3 ± 1.4	0.940	3.3 ± 0.6	4.4 ± 1.3	0.414
pathway	number of test probes to E1	2	#	10.5 ± 2.6	18.5 ± 2.6	0.041	6.5 ± 1.6	2.5 ± 0.4	0.038
	minimum C prior to E1	3	min	7.1 ± 0.8	15.0 ± 1.6	0.003	7.4 ± 1.1	4.7 ± 0.6	0.032
	total time of F	4	min	0.0 ± 0.0	11.0 ± 3.7	0.023	0.0 ± 0.0	3.9 ± 2.7	0.274
	number of F	5	#	0.0 ± 0.0	1.0 ± 0.3	0.008	0.0 ± 0.0	0.1 ± 0.1	0.263
phloem	time from 1st probe to 1st E1	6	min	60.0 ± 12.6	136.5 ± 18.5	0.019	132.7 ± 22.6	95.6 ± 14.5	0.115
	time from 1st probe to 1st sE2	7	min	128.5 ± 18.9	283.4 ± 41.9	0.018	146.8 ± 30.1	136.5 ± 29.8	0.414
	number (%) of aphids with sE2	8	#	18 100%	10 67%	0.013	16 100%	15 100%	1.000
	total time of E2	9	min	97.5 ± 10.4	114.7 ± 25.2	0.699	244.8 ± 33.8	156.9 ± 36.9	0.089
	average E2 duration	10	min	7.8 ± 2.4	13.9 ± 5.2	0.380	132.8 ± 36.7	77.5 ± 37.2	0.066
xylem	total time of G	11	min	15.2 ± 5.8	11.8 ± 3.3	0.573	60 ± 7.9	27.2 ± 5.9	0.005
	number of G	12	#	1.2 ± 0.3	1.0 ± 0.0	0.810	2.4 ± 0.3	1.3 ± 0.2	0.009

EPG recording with each aphid was conducted for 8 h. Values are means ± SE of EPG parameters. Mann-Whitney U tests were used to determine the significant difference between the activities of aphids on the mutant and the wild type plants. Fisher's exact test was applied to analyse the number of aphids that had shown sE2. Grey boxes indicate a significant difference ($P < 0.05$).

Brevicoryne brassicae performance on mutant 3646

Based on the EPG results, that suggest that *IRM1* confers a mechanical barrier against the generalist aphid *M. persicae*, we hypothesized that the *IRM1* resistance is general and affects other aphid species as well. To test this hypothesis, we monitored population development of the specialist aphid *B. brassicae* on mutant 3646. The total number of *B. brassicae* aphids was significantly lower on mutant 3646 than on wild type plants 14 days after infestation, with an average of seven aphids on mutant 3646 and 19 aphids on the wild type (Independent sample *t* test, $P < 0.001$, $n = 15$).

Discussion

Overexpression of *IRM1* increases aphid resistance in *A. thaliana*.

We identified At5g65040 as the gene responsible for the increased resistance towards *M. persicae* in mutant 3646 (Chen et al., 2012) and named it *Increased Resistance to Myzus persicae 1* (*IRM1*). In this mutant *IRM1* is constitutively expressed due to the insertion of a 35S promoter upstream of the gene. The negative effect of a constitutive overexpression of the *IRM1* gene on aphid population development was confirmed in transgenic lines that contained the cloned gene under the control of a CaMV 35S promoter in Col-0 background. Conversely, a T-DNA insertion mutant (40-KO), which did not show any expression of the *IRM1* gene, showed improved aphid performance. An analysis of gene expression profiles in publicly available microarray data sets using Genevestigator showed that *IRM1* expression is strongest in the xylem and very low in other plant tissues (https://www.genevestigator.com/; (Hruz et al., 2008)). Although *IRM1* has been predicted to encode a DUF581 domain containing protein (Lamesch et al., 2012), there is nothing known about the function of this gene.

Our data showed that the expression levels of *IRM1* differed among the four independent transgenic lines (in Col-0), but the reduced aphid number on these lines was comparable. In addition, the twofold increased *IRM1* expression in mutant 3646 compared with the wild type WS conferred a similar resistance level (Chen et al., 2012). These results indicate that the plant resistance conferred by constitutive overexpression of *IRM1* is not dependent on the expression of *IRM1* alone; after a certain transcript abundance is reached, additional transcripts do not increase resistance any further, suggesting that other factors become limiting.

The *IRM1* expression was shown to be induced in one microarray study with *M. persicae* infested *A. thaliana* Col-0 plants (De Vos et al., 2005), but not in others (Couldridge et al., 2007; De Vos and Jander, 2009). These conflicting results may be caused by the fact that the expression of *IRM1* is too low for a stable detection in a microarray study. We found *IRM1* expression to be suppressed in Col-0 upon aphid infestation whereas in WS it was initially induced, but suppressed afterwards. Such differences may result from the genetic differences among the two *A. thaliana* ecotypes in the basal defense to aphids (Ahmad et al., 2011).

Overexpressing *IRM1* causes difficulties for aphids to reach the phloem.

The electrical penetration graph (EPG) technique can reveal possible constraints that an aphid encounters while trying to feed on a plant (Tjallingii et al., 2010). The EPG results indicate that plant resistance conferred by overexpressing *IRM1* affects the aphid in its ability to reach the phloem (stylet pathway phase). All parameters that were used to describe this phase (Table 2) showed values that are significantly higher when *IRM1* was overexpressed. Contrarily, aphids on the *IRM1* knock out mutant could penetrate the plant tissue easier and had faster access to the phloem than aphids on the wild type. Furthermore, the aphids spent significantly less time in the xylem on the *IRM1* knock out mutant than

on the wild type, which indicates sufficient uptake of phloem sap (Spiller et al., 1990; Powell and Hardie, 2002) and also suggests that they encounter less resistance to access the phloem.

Overexpression of *IRM1* clearly disrupted the capability of *M. persicae* to reach sustained phloem sap ingestion as the tested aphids were either unable or needed double the time to reach this stage on the *IRM1* overexpression mutant 3646 compared to the wild type. Because this phase is needed to transmit persistent viruses (Stafford et al., 2012), the chance of virus transmission by aphids may be reduced due to *IRM1* overexpression. This is consistent with our previous observation in which the *IRM1* overexpression mutant was identified based on its lower percentage of virus infected plants (Chen et al., 2012).

To date, no information on a possible role of *IRM1* in xylem or other plant tissue is available. Considering the extremely reinforced cell walls in xylem (Karam, 2005), we speculate that *IRM1* overexpressing plants may have enhanced mechanical barriers that hamper penetration of plant tissue by aphids. This speculation is supported by the fact that *IRM1* overexpressing not only affects *M. persicae* but also adversely affect *B. brassicae*, an aphid species with the same feeding strategy but with a different host specialization. This suggests that the resistance acts as a mechanical barrier which is not aphid species specific. This aphid resistance mechanism in *A. thaliana IRM1* overexpressing plants is different from previously identified aphid resistance mechanisms, most of which are phloem based (Pegadaraju et al., 2007; Civolani et al., 2010; Zhu et al., 2011; Nalam et al., 2012).

Increased aphid resistance in *IRM1* overexpressing lines trades off with plant growth

It has been shown that plant resistance to insects and pathogens trades off with plant growth (Herms and Mattson, 1992; Bostock, 2005). In our study, we also see that *A. thaliana* lines constitutively overexpressing *IRM1* have an increased resistance to aphids, which is accompanied by poor plant growth. Similarly, constitutive activation of the jasmonic acid and ethylene pathway in *A. thaliana* mutant *cev1* increases resistance to aphids and pathogens but results in dwarf growth (Ellis et al., 2002). Also, the constitutive expression of a proteinase inhibitor that is induced in wild type plants by attackers in *Nicotiana attenuata*, leads to a significant reduction in plant growth (Zavala and Baldwin, 2006).

Conclusions

Constitutive overexpression of *IRM1* results in mechanical barriers that make it difficult for *M. persicae* to reach the phloem and subsequently reduces its population size. Overexpression of *IRM1* in *A. thaliana* also affects *B. brassicae* and may affect other phloem-feeding insects as well. A reduced capability to reach the phloem most likely reduces the transmission of persistent viruses. Increased aphid resistance in *IRM1* overexpressing *A. thaliana* plants is accompanied with reduced plant growth. Future experiments on the protein encoded by the *IRM1* gene, e.g. subcellular localization as well as its activity in plants and aphids, will help to provide functional insight into the role of *IRM1* in planta. This will lead to a better understanding of plant-aphid interactions on the molecular level.

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Supporting information

Supplemental Figure 1

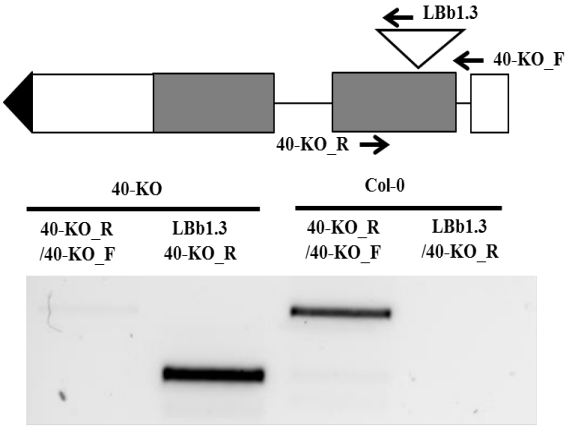

Diagram of the At5g65040 gene indicating position of the T-DNA insert (up part) and confirmation of the homozygous presence of the T-DNA in SALK_106042 (40-KO) (bottom part). Open boxes represent 5' UTR and 3'UTR; lines represent introns, grey boxes represent exons, black triangle at the end of the gene indicates the gene orientation. Inverted triangle represents T-DNA; arrows represent the gene specific primers and T-DNA left border primer. The primer combinations used for amplification are indicated above the gel lanes.

Chapter 5

Constitutive overexpression of the pollen specific gene *SKS13* in leaves enhances aphid resistance in *Arabidopsis thaliana*

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Submitted as Chen et al (2013)

Abstract

With the aim to identify genes that can increase plant resistance towards aphids, we previously screened an *Arabidopsis thaliana* activation tag mutant collection. Activation tag mutants display a gain-of-function phenotype, which results from the overexpression of tagged genes by a tetramer of the *Cauliflower mosaic virus* (CaMV) 35S promoter. From the *A. thaliana* mutant collection we identified several mutants on which aphid population development was reduced. Here we show that *SKU5 SIMILAR 13* (*SKS13*), a gene normally expressed in pollen only, is the gene responsible for the enhanced aphid resistance in one of these mutants. Aphid resistance conferred by overexpression of *SKS13* was confirmed in transgenic *A. thaliana* plants in which the cloned *SKS13* was expressed under control of the CaMV 35S promoter. Electrical penetration graph analysis of the aphid feeding behavior on *SKS13* overexpressing plants indicated that the increased resistance was phloem based. This resistance affected both *Myzus persicae* and *Brevicoryne brassicae* and most probably results from an accumulation of reactive oxygen species in the *SKS13* overexpressing plants. Furthermore, overexpression of *SKS13* in transgenic *A. thaliana* plants activated the jasmonic acid pathway. Taken together we show that *SKS13* is normally not expressed or induced by aphid infestation in leaves of Wassilewskija and Columbia-0, but overexpressing this gene in all plant tissues leads to an increased resistance against aphids.

Key words

phloem-feeding insect, *Myzus persicae*, *Brevicoryne brassicae*, activation tag mutant, overexpression, electrical penetration graph (EPG), reactive oxygen species (ROS), jasmonic acid (JA)

Introduction

Aphids have developed a sophisticated feeding strategy (Tjallingii, 1988). Their stylets penetrate plant tissue and puncture cells along the intercellular pathway towards the phloem (Tjallingii, 2006). Once an aphid establishes a feeding site it can feed from the phloem for hours or even days (Tjallingii, 2006). To facilitate the probing and feeding processes, aphids continuously secrete saliva into the plant tissue to degrade cell walls and to overcome occlusion of the feeding site (Will et al., 2009; Moreno et al., 2011). However, the secretion of saliva is also thought to activate plant defense (Maffei et al., 2006; Harmel et al., 2008). Plants have evolved a series of defense traits to directly affect the aphid's behavior. These defenses include physical and chemical traits that can be constitutively present of induced upon aphid attack (Howe and Jander, 2008). Physical traits, such as hairs and glandular trichomes, hinder aphid settling on a plant (Alvarez et al., 2006). Chemical traits include the production of secondary metabolites and proteins that are repellent or toxic to aphids thereby affecting aphid performance (Halkier and Gershenzon, 2006.) For example, the brassicaceous-specific secondary metabolites glucosinolates have been shown to negatively affected the performance of the generalist aphid *Myzus persicae* (Kim et al., 2008).

The activation of plant hormone pathways, especially jasmonic acid (JA), salicylic acid (SA) and ethylene (ET), plays an important role in plant defense against aphids (Wu and Baldwin, 2010; Morkunas et al., 2011). These pathways interact in a network, regulating the expression of specific groups of defense-related genes (Robert-Seilaniantz et al., 2011). Although all pathways are involved in defense, the JA pathway is thought to be the most effective against aphids (Thompson and Goggin, 2006) (Zhu-Salzman et al., 2005). Constitutive activation of the JA pathway in an *Arabidopsis thaliana* mutant leads to enhanced aphid resistance, whereas blocking the JA pathway results in aphid susceptibility (Ellis et al., 2002).

An increasing body of evidence suggests that reactive oxygen species (ROS) can play a role in plant defense towards aphids as well (Maffei et al., 2007; Kerchev et al., 2012). For example, an early accumulation of ROS upon Russian wheat aphid infestation was suggested to be a defense response in aphid resistant wheat (Moloi and van der Westhuizen, 2006). In contrast, an increasing concentration of ascorbic acid, a compound that is capable of reducing ROS, leads to an enhanced aphid fecundity (Kerchev et al., 2012), further underpinning the role of ROS in plant defense towards aphids. Moreover, ROS can act as signaling molecules, along with JA to confer aphid resistance (Miller et al., 2009).

It has been shown that certain genes, for instance *IQD1*(*IQ-Domain1*) and *MPL1* (lipid biosynthesis related genes *Myzus persicae –induced lipase 1*) can confer plant resistance to insects when their level of expression is increased or the location of expression is changed (Levy et al., 2005; Zhang et al., 2006; Louis et al., 2010). Such genes may be identified by screening activation tag mutant collections for insect resistance (Levy et al., 2005; Chen et al., 2012). In these mutants, tagged genes are overexpressed by a tetramer *Cauliflower mosaic virus* (CaMV) 35S enhancer adjacent to the natural promoter, resulting in a dominant gain-of-function phenotype (Marsch-Martinez et al., 2002). By screening such an *A. thaliana* mutant collection, we have identified several mutants with increased aphid resistance (Chen et al., 2012). In the present paper we characterize one of these mutants, leading to the identification of *SKU5 SIMILAR 13* (*SKS13*) as the gene responsible for the increased aphid resistance. We suggest that aphid resistance conferred by overexpression of *SKS13* is mediated by the accumulation of ROS in leaves, possibly through affecting the JA pathway.

Materials and Methods

Insect rearing

Myzus persicae was reared in cages on Chinese cabbage (*Brassica rapa* L. ssp. *Pekinensis* cv. Granaat). *Brevicoryne brassicae* was reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus) at the Department of Entomology, Wageningen University. Both rearings were maintained in an acclimatized room with a relative humidity of 60-70%, a temperature of 20 ± 2 ºC and an 18:6 L:D photoperiod. For all experiments, only apterous aphids were used.

Plant material and growth conditions

Mutant 3790, , was previously identified from an *A. thaliana* (accession WS) activation tag library as a mutant on which aphids showed a reduced population development (Chen et al., 2012). Seeds of this mutant and *A. thaliana* accession WS were obtained from the library present at Wageningen UR Plant Breeding (Marsch-Martinez et al., 2002). Seeds of *brl3-2* and *brl3-3* mutants and their corresponding wild type accession Col-0 were kindly provided by Prof. S.C. de Vries, Laboratory of Biochemistry Wageningen University (Caño-Delgado et al., 2004). To induce germination, seeds were placed at 4ºC in the dark for 3 days under high humidity. Subsequently, seeds were transferred to potting compost (Lentse Potgrond®) and plants were cultivated in a climate chamber with a 6:18 L:D photoperiod. The temperature was maintained at 20 ± 2 ºC during the day and 18 ± 2 ºC during the night (60-70% relative humidity). Plants were watered every other day and no pest control was applied.

Inverse PCR (iPCR)

Genomic DNA of leaves collected from mutant 3790 was extracted using the DNeasy Plant Mini kit (Qiagen). Isolated DNA was digested with restriction enzyme EcoRI (Thermo, product # ER0275) or BamHI (Thermo, product # ER0051) and subsequently ligated with T4 DNA ligase (Fermentas, product # EL0011). Five µl of ligated DNA was used as a template in an iPCR reaction that was performed in a total volume of 50 µl containing the Phusion™ enzyme (Finnzymes, Product codes: F-530S, 100U). All enzymes were used according to the supplier's manuals. Primers were designed with Primer-3-Plus (Untergasser et al., 2007). For transposon flanking sequence isolation, primers Bar_F and Bar_R were designed based on the sequences of the *BAR* gene that is located on the transposon (Figure 1B, Table 1). For T-DNA flanking sequence isolation, primers (T-DNA LB_F and T-DNA LB_R) were designed based on the sequences of the T-DNA left border (Figure 1B, Table 1) since the right border of T-DNA is commonly lost upon integration (Weigel et al., 2000). The following iPCR program was used: 30 seconds at 98 ºC followed by 35 cycles of 98 ºC for 10 sec, 64 ºC for 10 sec, and 72 ºC for 3 min with a final extension at 72 ºC for 10 min. PCR products were sequenced and then blasted against the *A. thaliana* genome (http://www.arabidopsis.org/; (Altschul et al., 1990).

Time course experiment of aphid infestation

To investigate gene induction of *A. thaliana* WS and Col-0 in response to aphid infestation four-week-old plants were infested with 15 randomly selected aphids. Leaf material was collected at zero, six and 24 hours after the start of the aphid infestation. Aphids were gently brushed away from the leaf tissue. Uninfested *A. thaliana* plants were also brushed. Leaf material was immediately flash frozen in liquid nitrogen and stored at -80 ºC until use.

RNA isolation and cDNA synthesis

Total RNA of leaves was extracted using the RNeasy Plant mini kit (Qiagen). One µg of total RNA was treated with DNaseI (Invitrogen) according to the manufacturer's instructions. DNA-free total

RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). Synthesised cDNA was diluted 20 times.

Table 1. Primer sequences used in this study

name	purpose	sequence (5'--3')
Bar_F	iPCR	GCGTCGTTCTGGGCTCATGGT
Bar_R	iPCR	CTGGCAGCTGGACTTCAGCCTG
T-DNA_LB_F	iPCR, Semi-qPCR	CCCGTCTCACTGGTGAAAAGAA
T-DNA_LB_R	iPCR	ATTCGGCTATGACTGGGCACA
BRL3_F	qPCR	GGACATACCCGGGAGTACCT
BRL3_R	qPCR	CCCGTGTCTCAGATTTTGGT
SKS11_F	qPCR, Semi-qPCR	CAACTGTGGAATGTGGAACG
SKS11_R	qPCR	GGTGACAAGACACTCGCGTA
SKS13_F	qPCR	GAGCTACGAAGGAAGCAACG
SKS13_R	qPCR	CACTGGCGGTTAAGTTCCAT
LOX2_F	qPCR	AGATTCAAAGGCAAGCTCCA
LOX2_R	qPCR	ACAACACCAGCTCCAGCTCT
VSP2_F	qPCR	TACGAACGAAGCCGAACTCT
VSP2_R	qPCR	GGCACCGTGTCGAAGTCTAT
PDF1.2_F	qPCR	CACCCTTATCTTCGCTGCTC
PDF1.2_R	qPCR	GCACAACTTCTGTGCTTCCA
PAD4_F	qPCR	GTTCTTTTCCCCGGCTTATC
PAD4_R	qPCR	CGGTTATCACCACCAGCTTT
PR1_F	qPCR	GGCCTTACGGGGAAAACTTA
PR1_R	qPCR	CTCGCTAACCCACATGTTCA
ERF1_F	qPCR	CTTCCGACGAAGATCGTAGC
ERF1_R	qPCR	TCTTGACCGGAACAGAATCC
ACTIN8_F	qPCR	GATGGAGACCTCGAAAACCA
ACTIN8_R	qPCR	AAAAGGACTTCTGGGCACCT
BRL3_GSP	Semi-qPCR	AGACAACAACCTTGTGGGATG
Int2	Semi-qPCR	CAGGGTAGCTTACTGATGTGCG
AttB1_SKS13_F	Construction of transgenic plants	GGGGACAAGTTTGTACAAAAAAGCAGGCT CGAGCGAGAGAGATTCAAAAAT
AttB2_SKS13_R	Construction of transgenic plants	GGGGACCACTTTGTACAAGAAAGCTGGGT TCCTCTCTGGATTGAACAATGA
AttB1_F	Construction of transgenic plants	GGGGACAAGTTTGTACAAAAAAGCAGGCT
AttB2_R	Construction of transgenic plants	ACCACTTTGTACAAGAAAGCTGGGT

Quantitative RT-PCR

Gene specific primers were designed with Primer-3-Plus software (Untergasser et al., 2007) and are listed in Table 1. The *ACTIN8* (At1g49240) was used as a reference to normalize gene expression across the samples (Iven et al., 2012). Quantitative RT-PCR was performed in a total volume of 10 µl containing 2 µl cDNA, 1.5 µl of each gene-specific primer (0.5µM), and 5 µl SYBR Green Supermix Reagent (BioRad). Samples were designed in three biological replicates, with 17 individual plants pooled per replicate. Quantitative RT-PCR was performed in duplicate in a Real-Time Thermal Cycler (BioRad). The following program was used: 95 ºC for 3 min followed by 40 cycles of 95 ºC for 15 sec, and 60 ºC for 1 min. Threshold cycle (Ct) values were calculated by Optical system software, version 2.0 for MylQ (BioRad). The Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of the *ACTIN8* from the Ct value of the gene of interest. Normalized gene expression was computed as $2^{-\Delta ct}$ and Log_2-transformed prior to analysis. Independent-samples *t*-test or ANOVA followed by Tukey tests were used to determine the significance between genotypes ($P< 0.05$).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR reaction was performed in a total volume of 20 µl according to the manual of Phire® (Finnzymes, Product codes: F-122S). The PCR program consisted of 30 seconds at 98 ºC followed by 35 cycles of 98 ºC for 5 sec, 63 ºC for 5 sec, and 72 ºC for 30 sec with a final extension at 72 ºC for 3 min.

Construction of transgenic *A. thaliana* plants

To construct transgenic *A. thaliana* lines overexpressing *SKS13*, the coding region fragment of *SKS13* was amplified from Col-0 cDNA using primers AttB1_SKS13_F and AttB2_SKS13_R (Table 1) to facilitate gateway-compatible cloning. The PCR reaction was performed in a total volume of 50 µl containing the Phusion™ enzyme (Finnzymes, Product codes: F-530S, 100U). The following PCR program was used: 30 seconds at 98 ºC followed by 35 cycles of 98 ºC for 10 sec, 64 ºC for 10 sec, and 72 ºC for 3 min with a final extension at 72 ºC for 10 min. The resulting PCR product was cleaned from 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen) and sequenced for verification. The verified coding region fragment of *SKS13* was transferred into donor vector pDONR207 using the Gateway® BP Clonase™ II enzyme mix (Invitrogen) to generate entry vector pDONR207::*SKS13*. The entry vector was subsequently cloned into Gateway destination vector pFAST-R02 (Shimada et al., 2010) using the Gateway LR® Clonase™ II enzyme mix (Invitrogen) to generate the expression construct pFAST-R02-*SKS13* in which *SKS13* is under the control of the CaMV 35S promoter. The construct was transformed into *E. coli* and transformants were checked by colony PCR using primers AttB1_F and AttB2_R (Table 1). After verifying the accuracy of the coding region fragment of *SKS13*, the construct was transformed into *Agrobacterium tumefaciens* strain GV3101 (Hellens et al., 2000) by electroporation. *Agrobacterium* mediated transformation (Clough and Bent, 1998) was used to introduce the pFAST-R02-*SKS13* plasmid into Col-0 flowers. Seeds containing the construct were selected using fluorescence microscopy (Zeiss, SteREO Discovery.V8) (Shimada et al., 2010).

No-choice aphid assays

Synchronized one-day-old nymphs were placed on the middle of three-week-old plants fine brush. Each plant received one nymph and the total number of aphids was counted 14 days after infestation. The plants were randomly organized with 15 biological replicates per genotype Independent-samples *t*-test or ANOVA followed by Tukey tests were used to determine the significance between genotypes ($P< 0.05$).

Electrical penetration graph

The electrical penetration graph (EPG) technique (Tjallingii, 1990) was employed to monitor aphid feeding behavior. A gold wire (diameter 20 µm) was attached onto the dorsum of young adult aphids using conductive water-based silver glue. The wired aphid was placed on the nature leaf of a five-week-old plant that was connected to a recording system via a copper electrode in the soil (Tjallingii, 2006). All tested aphids stayed at the underside of the leaf. The EPGs were recorded at 22 ºC with constant light for 8 hours. The EPG data were analyzed using the PROBE 3.0 software (Wageningen University, the Netherlands) to distinguish the various waveforms. Waveform C represents the pathway phase, when the aphid stylet is penetrating through the leaf tissue; waveform E2 represents phloem sap ingestion; Waveform F is associated with derailed stylet mechanics or penetration difficulties; and waveform G indicates active uptake of water from the xylem (Tjallingii, 1990). Parameters were analyzed individually for each aphid. At least 15 recordings of individual aphids (one aphid per plant) were obtained for each genotype. The Mann-Whitney U and Fisher exact test were used to determine the significance difference between genotypes ($P< 0.05$).

Determination of ROS accumulation

To visualize ROS, leaves were cut from four-week-old plants and submerged overnight in HCl solution containing 1 mg mL^{-1} 3-3'-diaminobenzidine (DAB), pH 3.7 (Orozco-Cardenas and Ryan, 1999). Chlorophyll was extracted with 96% ethanol overnight at room temperature. Leaves were subsequently photographed in 80% glycerol.

Results

Phenotypic characterization of mutant 3790

Mutant 3790 was previously identified as an *A. thaliana* activation tag mutant on which *Myzus persicae* shows a longer pre-reproductive period and produces smaller numbers of offspring than on wild type Wassilewskija (referred to as WS hereafter) (Chen et al., 2012). Compared to WS, mutant 3790 has smaller and darker green colored leaves (Figure 1A), shows a delayed flowering, a reduced height of the main stem and an increased number of lateral branches.

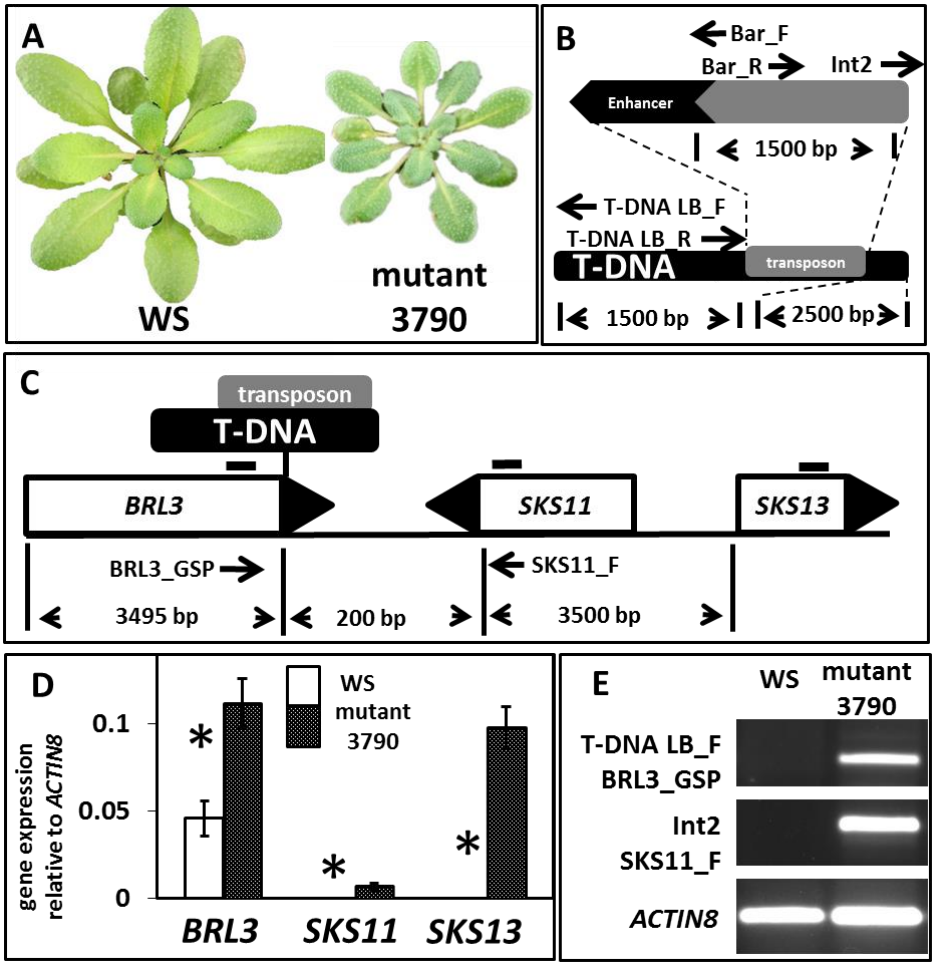

Figure 1. Phenotype, location of the T-DNA insert and expression analysis of flanking genes in mutant 3790. (A) Photographs of six-week-old Wassilewskija (WS) and mutant 3790. (B) Primers designed on T-DNA (black square) and transposon (grey square) for inverse PCR and semi-quantitative RT-PCR. The enhancer is illustrated as a black arrow on the left end of the transposon. The distance from the enhancer to the T-DNA border is indicated. (C) Position of the T-DNA insert containing the transposon in *BRL3* in mutant 3790. Black triangles at the end of the genes indicate the gene orientation. Short lines above the genes represent the primers used for quantitative RT-PCR

whereas arrows below the genes represent primers used for semi-quantitative RT-PCR. The distance from a gene to the T-DNA insert is indicated below the horizontal line. Diagram is not drawn to scale. (D) Quantitative RT-PCR of the genes flanking the T-DNA. Values are the means ± standard deviation of three biological replicates. The star indicates a significant difference between bars within a pair (Independent-samples *t*-test, $P < 0.05$). (E) Semi-quantitative RT-PCR of the genes flanking the T-DNA by using the combinations of primers indicated in (B) and (C).

Identification of the gene conferring increased resistance to aphids

Using inverse PCR (iPCR) we could determine that the transposon is still on the T-DNA (Figure 1B), and located on chromosome 3 at position 4,350,852 (according to the TAIR website; http://www.arabidopsis.org) in the 3'UTR region of the *Brassinosteroid Receptor Like* gene (*BRL3*, At3g13380; Figure 1C). Additionally, two other genes, *SKU5 Similar 11* (*SKS11*, At3g13390) and *SKU5 Similar 13* (*SKS13*, At3g13400) are located within a distance of approximately 8 kb of the enhancer (Figure 1B, C), a distance over which the enhancer can effectively activate the expression of genes (Ichikawa et al., 2003). To determine whether the transcript levels of these three genes were affected by the enhancer, we first performed quantitative RT-PCR and demonstrate that the transcript levels of all three candidate genes were significantly higher in mutant 3790 than in WS leaves (Figure 1D). The transcript level of *BRL3* was two-fold higher in mutant 3790 than in WS (Figure 1D). No transcripts of *SKS11* and *SKS13* were detectable in WS but they could clearly be detected in mutant 3790 (Figure 1D).

Considering that the position of the T-DNA containing the transposon is in the 3'UTR region of *BRL3*, the detected higher level of transcript of this gene in mutant 3790 is most likely due to an antisense transcript driven by the promoter on the T-DNA. Support for this was obtained from semi-quantitative RT-PCR in which a *BRL3* gene specific primer (*BRL3*_GSP) was used in combination with a T-DNA left border specific primer (T-DNA LB_F) (Figure 1B, C). As shown in figure 1E, there was no amplification with this primer pair in WS samples, whereas a clear product was obtained in mutant 3790 samples. As the transcription of antisense RNA can lead to gene silencing (Di Serio et al., 2001), we determined whether aphid resistance of mutant 3790 is due to the impaired expression of *BRL3*. To this purpose, we performed no-choice aphid assays and compared aphid population development on *BRL3* knock out mutants *brl3-2* and *brl3-3* with that on wild type Columbia-0 (referred to Col-0 hereafter) (Caño-Delgado et al., 2004). As shown in figure 2, the numbers of aphids on these mutants did not differ from those on Col-0 (Independent-sample *t* test, $P > 0.05$), indicating that *BRL3* is not the gene responsible for the increased aphid resistance in mutant 3790.

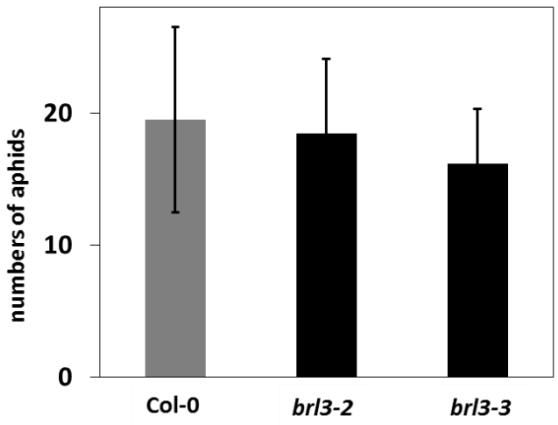

Figure 2. Aphid performance on *BRL3* knock out mutants. Values are the means ± standard deviation of 15 biological replicates. There were no significant differences between genotypes (ANOVA followed by Tukey tests, $P > 0.05$).

Similar to *BRL3*, the T-DNA containing the transposon was also downstream of *SKS11*, suggesting the presence of *SKS11* antisense transcripts as well. Indeed, we could amplify the antisense transcript of *SKS11* in mutant samples by using a *SKS11* gene specific primer (*SKS11*_F) combined with a transposon specific primer (Int2) (Marsch-Martinez et al., 2002) (Figure 1B, C), whereas WS samples showed no amplification (Figure 1E). Because *SKS11* was not expressed in leaves of aphid-free WS plants (Figure 1D), we performed a time course experiment to reveal whether *SKS11* is induced by aphid infestation. Induced expression of *Lipoxygenase 2* (*LOX2*) indicated an efficient aphid infestation (Moran and Thompson, 2001), but the expression of *SKS11* remained undetectable in WS leaves (Figure 3A). Therefore, a silencing effect of antisense transcript of *SKS11* cannot be the reason for the enhanced aphid resistance of mutant 3790.

Contrary to *BRL3* and *SKS11*, the position of *SKS13* is such that its expression can be activated by the enhancer located on the transposon. Because this gene was also not expressed or induced by aphid infestation in both WS and Col-0 leaves (Figure 3A, B), we did not evaluate aphid performance on *SKS13* knock out mutants. To confirm that overexpression of *SKS13* increased aphid resistance in mutant 3790, we constructed transgenic lines (G101, G102 and G103) in Col-0 by overexpressing *SKS13* under the CaMV 35S promoter. Compared to Col-0, these lines showed significantly higher expression of *SKS13* (Figure 4A) and lower numbers of aphids (Figure 4B). Similar to the comparison of mutant 3790 and WS, plants in these transgenic lines had smaller, rounder rosette leaves (Figure 4C), and longer time to flowering than Col-0. But the height of the main stem and the numbers of lateral branches of plants from these transgenic lines did not differ from Col-0.

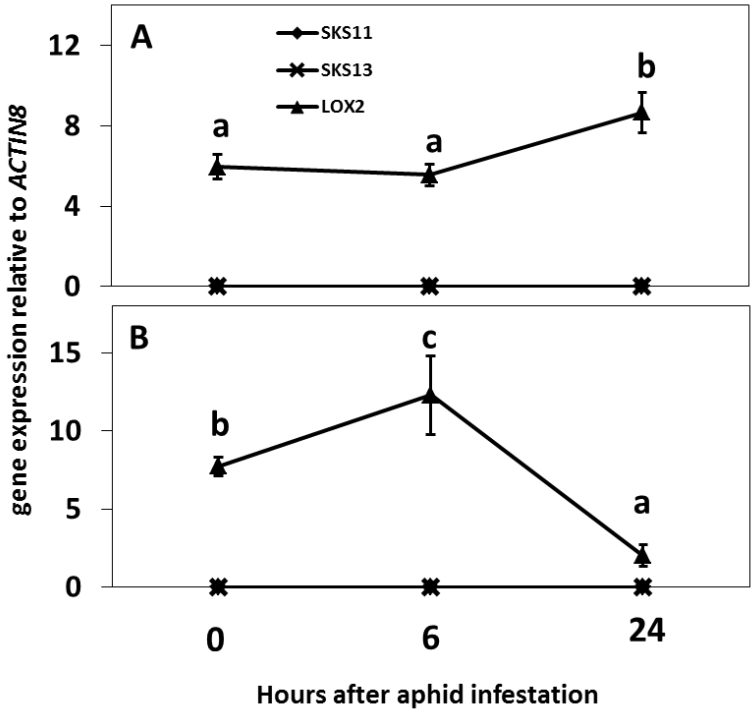

Figure 3. Gene induction upon aphid infestation in WS and Colombia-0. Quantitative RT-PCR of *SKS11*, *SKS13* and *LOX2* in WS (A) and Col-0 (B) plants at zero, six and 24 hours after the aphid infestation. Values are the means ± standard deviation of three biological replicates. Time points marked with different letters are significantly different from each other within one gene (ANOVA followed by Tukey tests, $P < 0.05$).

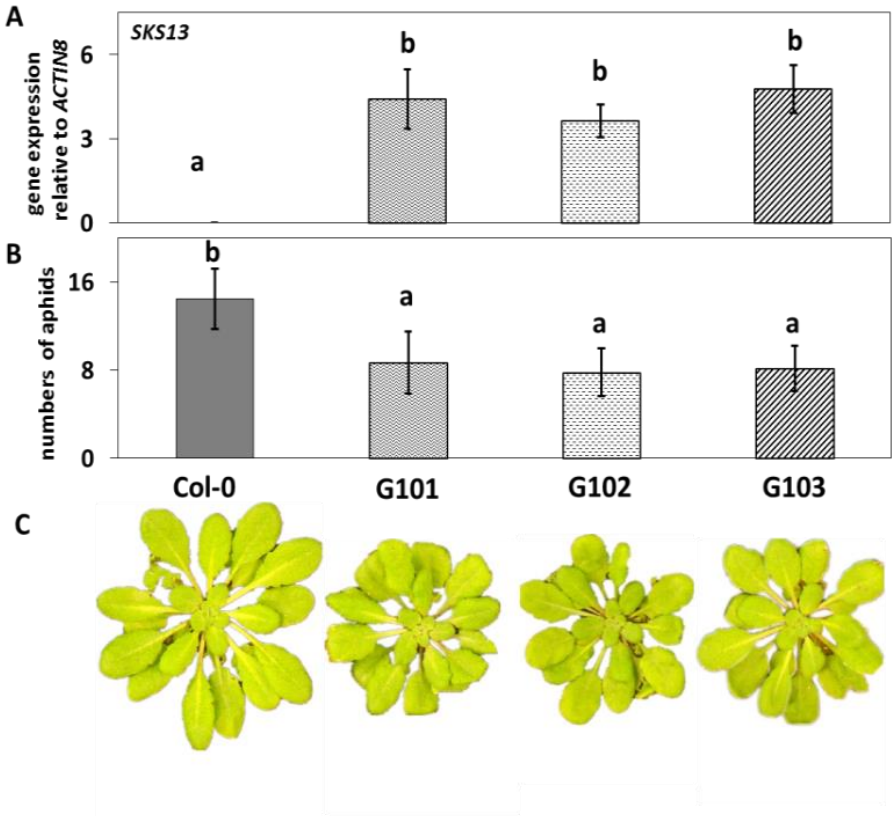

Figure 4. Gene expression analysis, aphid performance and phenotype of three independent *SKS13* overexpressing transgenic lines (A) Expression level of *SKS13* in leaves of Col-0 and transgenic lines (G101, G0102, G103). Values are the means ± standard deviation of three biological replicates (B) Aphid performance on Col-0 and the three transgenic lines. Values are the means ± standard deviation of 15 biological replicates. Bars marked with different letters are significantly different from each other (ANOVA followed by Tukey tests, $P < 0.05$). (C) Photographs of six-week-old plants of Col-0 and the three transgenic lines.

Aphid feeding behavior on mutant 3790

The electrical penetration graph (EPG) technique, in which the aphid and the plant are made part of an electrical circuit, registers signal waveforms reflecting aphid probing and feeding activities on a plant (Tjallingii, 2006). The technique can give indications on the location of plant resistance factors that affect aphid feeding behavior (Tjallingii et al., 2010). To reveal whether aphid feeding behavior was affected by overexpression of *SKS13* we compared EPG recordings of aphids on mutant 3790 and WS plants. As shown in figure 5A, no differences were observed for the total duration to the first probe, the total duration of the pathway phase and the total duration of the xylem phase. Aphids showed a significantly longer duration of the non-probe phase on mutant 3790 than on the WS (Figure 5A). Significant differences were also observed for phloem phase-related activities. Compared to WS, aphids on mutant 3790 needed double the amount of time to the first phloem phase, but spend only about one third of the total time in this phase (Figure 5A). Additionally, less aphids showed sustained phloem sap ingestion on mutant 3790 than on WS and the aphids that did show this activity on mutant 3790 spend smaller number of times in this phase than on WS (Figure 5B).

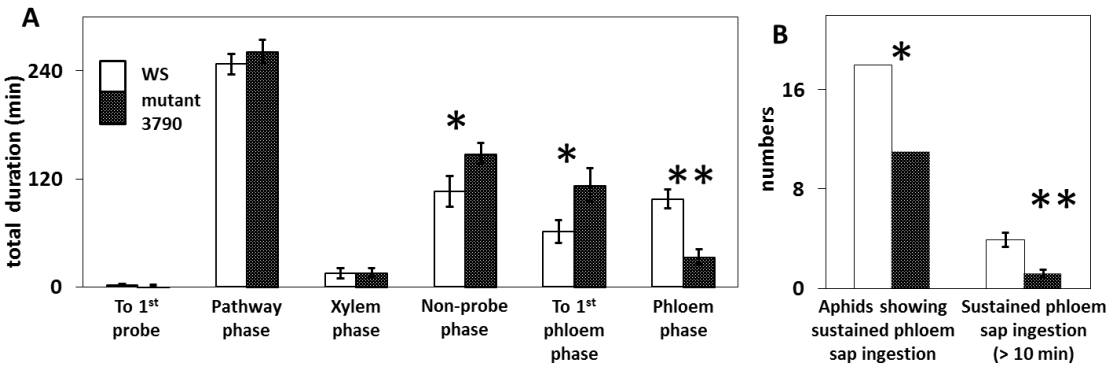

Figure 5. Electrical Penetration Graph (EPG) recordings. (A) Total duration (min) of a certain event. (B) The numbers of times that a certain event occurred. The EPG recording with each aphid was conducted for eight hours. Values are means ± standard error (SE) of EPG parameters from at least 15 replicates except for the parameters related with sustained phloem sap ingestion. (Fisher's exact test was applied for the numbers of aphids showing sustained phloem sap ingestion and Mann–Whitney U test was applied for the other parameters, The stars indicate significant differences between bars within pair *$P < 0.05$; ** $P < 0.01$).

Accumulation of reactive oxygen species in mutant line 3790

Gene *SKS13* has a putative function in oxidation/reduction reactions (Sedbrook et al., 2002; Lamesch et al., 2012) and its co-expressed genes function in ROS generation (Hruz et al., 2008; Wang et al., 2010). Therefore, we hypothesized that overexpression of *SKS13* may lead to an accumulation of ROS in leaves. To visualize ROS we used 3-3'-diaminobenzidine (DAB) staining on the leaves of WS, mutant 3790, Col-0 and transgenic line G101. Each leaf was injured by forceps to serve as a positive control for the DAB staining (Takahashi et al., 2011); Figure 6). In comparison to WS and Col-0 leaves, darker browning was observed in leaves of mutant 3790 and transgenic line G010, respectively (Figure 6).

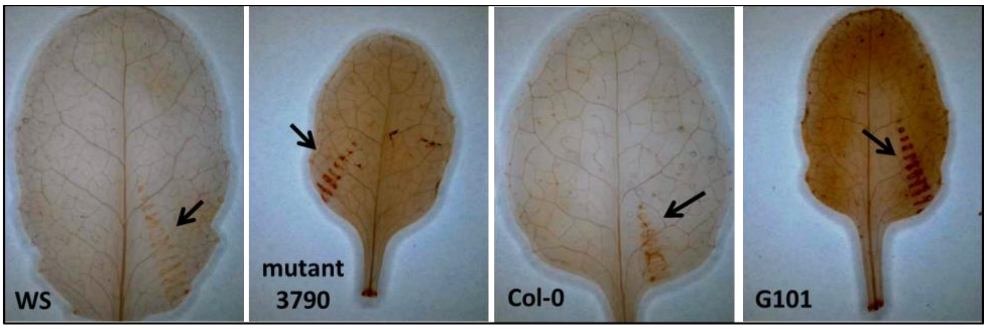

Figure 6. Accumulation of ROS in *SKS13* overexpressing plants. 3-3'-diaminobenzidine (DAB) staining of detached leaves from WS, mutant 3790, Col-0 and *SKS13* overexpressing transgenic line G101. An arrow pointed to the part of each leaf that was injured by forceps to serve as a positive control for the DAB staining.

Brevicoryne brassicae performance on mutant 3790

It has been suggested that the ROS accumulation plays a general role in plant defense against aphids (Moloi and van der Westhuizen, 2006; Miller et al., 2009). Therefore, we hypothesized that *SKS13* overexpressing plants would not only affect the generalist *M. persicae* but also other aphid species. This hypothesis was tested by infesting mutant 3790 and WS with the specialist *B. brassicae*. At 14

days after infestation, an average of four aphids was found on mutant 3790 and 18 aphids on the WS plants (Independent-sample *t* test $P < 0.001$, n = 15).

Expression of plant hormone pathway genes in *SKS13* overexpressing plants

To determine whether overexpression of *SKS13* affects the plant hormone pathways, we compared the expression level of JA-, SA- and ET-marker genes between mutant 3790 and WS, as well as between *SKS13* overexpressing transgenic lines and Col-0. The expressions level of the JA-marker genes *LOX2 (Lipoxygenase 2)*, *VSP2* (*Vegetative Storage Protein 2*) and *PDF1.2* (*Putative plant defensin 1.2*) as well as SA-marker genes *PAD4* (*Phytoalexin Deficient4*) and *PR1* (*Pathogenesis-related 1*) were not significantly different between mutant 3790 and the WS. However, the expression level of the ET-marker gene *ERF1* (*Ethylene response factor 1*) was significantly higher in mutant 3790 than in WS (Figure 7). Conversely, the *SKS13* overexpressing transgenic lines showed significant higher expression level compared to Col-0 for the JA-marker genes only (Figure 7).

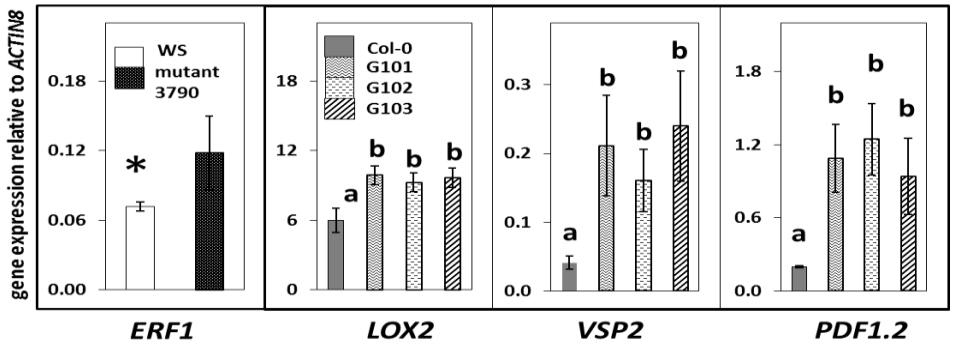

Figure 7. Expressions of ET and JA pathway marker genes in mutant 3790 and *SKS13* overexpressing transgenic lines. Values are the means ± standard deviation of three biological replicates. The star indicates a significant difference between mutant 3790 and the WS (Independent-samples *t*-test, $P < 0.05$). Bars marked with different letters are significantly different from each other (ANOVA followed by Tukey tests, $P < 0.05$).

Discussion

Overexpression of *SKS13* in leaves enhances aphid resistance in *A. thaliana*

Mutant 3790 was previously identified as an *A. thaliana* mutant with increased resistance against *M. persicae* (Chen et al., 2012). In the present paper we show that the constitutive overexpression of *SKS13* increased aphid resistance. The reduced aphid population development due to the constitutive overexpression of *SKS13* was confirmed in transgenic plants that embraced the *SKS13* under the control of CaMV 35S promoter.

An analysis of expression profiles in publicly available microarray data sets revealed that *SKS13* is exclusively expressed in pollen (https://www.genevestigator.com/; (Hruz et al., 2008). This is in agreement with our observation that *SKS13* was not expressed in leaves of WS or Col-0. We also demonstrated that the expression of *SKS13* was not induced upon aphid infestation. This is consistent with previous microarray studies in which no induction of *SKS13* expression in *A. thaliana* after *M. persicae* infestation was found (Moran et al., 2002; De Vos et al., 2005; Kempema et al., 2007).

Overexpression of *SKS13* affects aphid feeding behavior probably due to ROS accumulation

Analysis of aphid feeding behavior by the EPG technique can provide insight into the plant resistance mechanisms (Tjallingii et al., 2010). The EPG results indicated that plant resistance conferred by overexpression of *SKS13* was phloem based. This was supported by the fact that the phloem phase of aphids on *SKS13* overexpressing plants was delayed in time and reduced in length. The phloem based resistance was further indicated by the reduced number of sustained phloem sap ingestions. As sustained phloem sap ingestion is required for the transmission of persistently transmitted viruses (Stafford et al., 2012), the phloem based resistance explains the decreased transmission of such a virus, i.e. *Turnip yellows virus*, as previously observed in mutant 3790 (Chen et al., 2012).

To uncover the role of *SKS13* in the phloem based plant resistance to aphids, we explored the possible biological function of this gene. As structurally related to multiple-copper oxidases, ascorbate oxidases and laccases, *SKS13* has been suggested to have a putative function in oxidation/ reduction reactions (Sedbrook et al., 2002) (Lamesch et al., 2012). Furthermore, *SKS13* is co-expressed with genes involved in ROS generation (https://www.genevestigator.com/ (Hruz et al., 2008; Wang et al., 2010)). Therefore we hypothesized that constitutive overexpression of *SKS13* results in an accumulation of ROS in leaves, and confirmed this by DAB staining the leaves of *SKS13* overexpressing plants. The effect of ROS accumulation on aphid feeding behavior has also been shown for a triticale cultivar with a high concentration of ROS on which aphids displayed a reduced time in the phloem phase and a prolonged time in the non-probe phase (Łukasik et al., 2012). This is similar to our observations of aphid feeding behavior on *SKS13* overexpressing plants. The accumulation of ROS was suggested to play a role in plant resistance to several aphid species (Moloi and van der Westhuizen, 2006; Łukasik et al., 2012). This is in line with our results, as aphid resistance on *SKS13* overexpressing plants not only affected *M. persicae* but also *B. brassicae* performance. Besides enhancing aphid resistance, excessive ROS can damage proteins, lipids and nucleic acids and can eventual be harmful to plant growth (Apel and Hirt, 2004), thereby explaining the reduced size of *SKS13* overexpressing plants.

Overexpression of *SKS13* affects plant hormone pathways in *A. thaliana*

Several studies suggest that ROS tangle with plant hormone pathways, such as JA, SA and ET in plant defense to aphids (Argandoña et al., 2001; Mohase and van der Westhuizen, 2002; Moloi and van der Westhuizen, 2006; Miller et al., 2009). For instance, *A. thaliana RbohD* mutant, in which JA induced ROS accumulation does not occur, promotes a four times larger aphid population development than its

wild type Col-0 (Miller et al., 2009) (Maruta et al., 2011), suggesting that aphid resistance conferred by activation of the JA pathway is probably mediated by ROS accumulation. In our study, we observed an activation of the JA pathway in *SKS13* overexpressing Col-0 plants, as indicated by the significant higher expressions level of the three JA marker genes. This finding suggests that ROS interacts with JA in *SKS13* overexpressing Col-0 plants.

In mutant 3790, *SKS13* is overexpressed in the WS background and the ET pathway is activated instead of the JA pathway, which may be due to the genetic differences between Col-0 and WS in response to ROS accumulation (Ahmad et al., 2011). In addition to *SKS13*, the *BRL3* gene is most likely silenced in mutant 3790. However, the additional characteristics of mutant 3790 do not resemble the *brl3* mutant of which the phenotype does not differ from Col-0 in the height of the main stem and an increased numbers of lateral branches (Caño-Delgado et al., 2004). Most likely the additional phenotypic differences seen in mutant 3790 are caused by a second mutation which is not related to the transposon insertion in the 3' end of the *BRL3*. Alternatively the additional differences may be attributed to unknown interactions among BR, ET and ROS.

Conclusions

Overexpression of *SKS13* in *A. thaliana* leads to a reduced phloem feeding of *M. persicae*, which probably is due to accumulation of ROS in leaves. The reduced phloem feeding results in the suppression of the population development of *M. persicae* and also decreases the transmission of persistent viruses. Overexpression of *SKS13* in *A. thaliana* also affects *B. brassicae* and probably other phloem feeding insects. The increased resistance towards aphids in *SKS13* overexpressing *A. thaliana* plants reduces plant development.

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Chapter 6

General discussion

Xi Chen

Plants are under constant threat of insect attack. To defend themselves they have evolved defense mechanisms, which can be categorized into antixenosis and antibiosis (Howe and Jander, 2008). Unlike chewing insects that cause damage by removing large amounts of plant tissue, phloem feeding insects withdraw sap from phloem sieve elements, which generates relatively small direct damage to the host plants (Tjallingii and Esch, 1993; Miles, 1999; Kaloshian and Walling, 2005). They are a problem in crop plants because of the devastating viruses they transmit during the probing and feeding (Weintraub and Beanland, 2006). As a relatively large number of different viruses can be transmitted by one insect, which can rapidly adapt to a particular host plant (Harrison, 2002), it becomes an attractive strategy to search for resistance against the vector insects next to searching for resistance against individual viruses.

To develop plants resistant against phloem feeding insects, knowledge on plant-insect interactions and understanding of the resistance mechanism is a prerequisite. Transcription profile analysis of plants infested by phloem feeding insects revealed that hundreds, and in some cases even thousands, of genes are activated upon infestation (Zhu-Salzman et al., 2004; De Vos et al., 2005; Broekgaarden et al., 2007; Kusnierczyk et al., 2008). However, it is very time consuming to identify the genes that matter among such large amounts. Fortunately, with the development of transgenic techniques, plant genomes can be manipulated (Meissner et al., 2000; Bush et al., 2007; Haag, 2007; Qu et al., 2008). *Arabidopsis thaliana* as a model plant with short life cycle and small genome that can be easily manipulated has the most extensive mutant collections (Krysan et al., 1999; Weigel et al., 2000; Szabados et al., 2002; Alonso et al., 2003; Remy et al., 2005; Haag, 2007). The phenotype of these mutants is affected by either silencing or activating gene expression, thus providing ideal starting material to identify gene functions (Kuromori et al., 2009; O'Malley and Ecker, 2010). This strategy in combination with high-throughput phenotyping methods facilitated the identification of several new genes that confer resistance to biotic stresses (Aharoni et al., 2004; Pereira et al., 2007; Kondou et al., 2010). However, their use in the identification of genes that can confer increased resistance towards phloem feeding insects is still limited, which is mainly due to the lack of high-throughput phenotyping methods.

In this project, I used an *A. thaliana* activation tag mutant collection to study plant resistance towards aphids. I first established high-throughput screening methods to identify mutants with increased resistance to aphids. Then I characterized several identified mutants at the molecular level to reveal the underlying genes and resistance mechanisms.

High-throughput screening methods: a good start is half the battle

Plant resistance towards phloem feeding insects is normally quantified by regular observations of insect behavior and/or tedious counting of insect population development. Several screening methods have been developed for application in the field and in the laboratory. Natural infestation observations are commonly used in the field in which insects can randomly choose their favourite host plant (Stoner, 1990). However, the outcome of such natural infestations is highly dependent on the environmental conditions. For example, low levels of infestation or unfavourable weather conditions may lead to selection of material that in fact is not resistant at all. To prevent the identification of such false negatives more controlled conditions are needed. Plants may also be evaluated in a greenhouse (Sharma et al., 2005), which takes away most of the disadvantages of the open field trials. An even more artificial and controlled system are laboratory based assays, in which detached leaf or leaf disk assays are applied (Firdaus et al., 2011; Maharijaya et al., 2011). However, also these have their challenges as they require extra work to prepare the detached plant leaves and maintain the leaf

freshness. With none of these methods it is feasible to evaluate large numbers of accessions for increased aphid resistance. Therefore, there is still a need for good high throughput screening methods.

Virus transmission assays

Aphids are effective vectors of viruses (Brault et al., 2010), which is the main reason to search for aphid resistance. For the transfer of circulative viruses the aphids needs to feed from the phloem, as just probing the plant is not sufficient to transmit this type of virus (Ng and Perry, 2004). I have developed a screening method using the absence of a circulative virus *Turnip yellows virus* (TuYV) to indicate plant resistance towards the vector aphids. In this virus transmission assay, I directly transferred virus infected aphids onto each test plant and determined the virus infection of the plant two weeks after infestation. I validated this screening method using an *A. thaliana* activation tag mutant collection and identified nine mutants with reduced virus transmission out of 5160 mutants. All of them were confirmed as mutants showing increased aphid resistance whereas none of them were virus resistant (Chapter 3).

The success of this screening method is mainly attributed to the principle it is based on. Plant resistance traits affect aphid feeding behavior and consequently the efficiency of virus transmission into plants. For instance, the resistance traits of tomato wild relatives affect whitefly preference, their feeding behavior and also reduce the spread of viruses (Rodriguez-Lopez et al., 2011). The single dominant gene *Vat* (*Virus Aphid Transmission*) confers aphid resistance in melon and subsequently reduces aphid mediated virus transmission (Sarria et al., 2008). Not all viruses are equally suitable as indicator of plant resistance to insects. As mentioned, I used a circulative virus because it can only be transmitted during prolonged aphid feeding from the phloem sieve element (Hogenhout et al., 2008). A non-circulative virus can be transmitted by a short probe of an aphid (Pirone and Blanc, 1996). Using this type of virus will most likely abolish the chance of identifying putatively resistant candidates, especially the candidates that show a partial resistance, as the selection pressure imposed by the non-circulative virus is extremely high.

An advantage of the virus based screening method is that it does not require tedious counting of aphids but the plant resistance is indicated by the absence of virus. In our case I had to determine the presence or absence of the virus by ELISA, since *Turnip yellows virus* (TuYV) virus does not show symptoms on *A. thaliana*. An alternative would be to use PCR to determine the presence or absence of the virus. However the advantage of using a virus that shows phenotypic effects is that one can directly score the symptoms on a particular plant.

A high stringency of the screening method was ensured by using four virus infected aphids, a prolonged time for virus transmission (five days) and virus development (two weeks) (Chapter 3). On the one hand these parameters ensured that almost all putative mutants selected were indeed more resistant to aphids than the wild type. On the other hand it may have led to discarding many possibly interesting mutants. In this project, I screened more than 5000 activation tag mutants out of which nine were identified with increased insect resistance. However, it may be good to try to reduce the selection pressure, e.g. finding a better balance between false negative and false positive results. This may be achieved by using less virus infected aphids, shorter time for virus transmission (less than five days) and/or include more individual plants of each mutant in the initial screening.

Identifying genes involved in insect resistance using activation tag mutants

Activation tag mutant collections are good resources to identify gene function

Activation tag mutants and knockout mutants can be used to elucidate gene functions (Kondou et al., 2010; O'Malley and Ecker, 2010). In comparison to knockout mutants activation tag mutants have several advantages. First, in the *A. thaliana* activation tag mutants the expression of genes is brought under control of the CaMV 35S promoter, which ensures constitutive expression in almost all plant tissues throughout the whole plant life cycle (Lee et al., 2007). This leads to big differences for the genes that are temporarily expressed or in specific tissues only. By expressing them in leaves it is possible to study their putative role in plant-insect interactions. Gene *SKS13* (Chapter 5) is a nice example of this. Without an activation tag library this gene would never have been identified as a gene that may play a role in increasing aphid resistance. On the contrary, knockout mutants of *SKS13* would show no resistance phenotype as the gene is not expressed in leaves of wild type plants. Second, the dominant gain-of-function phenotype of activation tag mutants is displayed in both heterozygous and homozygous state. For a screening, this maximizes the chance to identify the phenotype when a gene is affected (Chapter 2 and 3). The nowadays available T-DNA insertion knockout mutant collections contain both heterozygous and homozygous insertion mutants (Krysan et al., 1999; Radhamony et al., 2005). Mutants with heterozygous insertion do not show any phenotypes, reducing the chance of identification (O'Malley and Ecker, 2010). It is true that loss-of-function of certain genes can affect insect performance and may also lead to increased resistance. For instance, the expression of *LOX5* facilitates aphid feeding, while the T-DNA knockout mutant *lox5* showed elevated resistance (Nalam et al., 2012). Although some knockout mutants may also be found when an activation tag library is used, as the T-DNA construct may actually insert into a gene and cause a knockout (Qu et al., 2008).

Furthermore, T-DNA knockout mutants cannot be used to discover genes that are present redundantly because the loss-of-function of one gene may be compensated by others (Krysan et al., 1999). In addition, genes involved in early embryo development that are inactivated by homozygous T-DNA insertion may lead to embryo lethality, therefore the gene function analysis cannot be carried out (Frans et al., 2001). These problems of T-DNA knockout mutants can be avoided by using activation tag mutants showing dominant gain-of-function phenotypes.

Increased plant resistance to insects by constitutive overexpression of genes

Following the identification of several mutants with enhanced resistance towards aphids I have further characterized three mutants and identified two genes *Increased Resistance* to *Myzus persicae 1* (*IRM1*; Chapter 4) and *SKU5 Similar 13* (*SKS13*; Chapter 5) that can enhance plant resistance to aphids when they are constitutively (over)expressed in leaves. I did not manage to isolate the flanking sequences of T-DNA which contains the enhancer in mutant 435 (Chapter 2), therefore I have no information about the affected gene. Both genes *IRM1* and *SKS13* are not likely to be involved in plant defense responses in wild type plants. The *IMR1* gene is expressed at extremely low levels in most of the leaf tissue and only in the xylem high levels are measured (Chapter 4, https://www.genevestigator.com/; (Hruz et al., 2008). Xylem transports water and soluble minerals which are not desired by aphids. Passive drinking from xylem is only observed when aphids cannot feed from phloem sieve elements (Powell and Hardie, 2002; Pompon et al., 2011). I show that upon aphid infestation the changes in the expression level of *IMR1* are minor and differ between *A. thaliana* accessions (Chapter 4). A defense response conferred by gene *SKS13* in wild type plants is even more unlikely since this gene is exclusively expressed in pollen (https://www.genevestigator.com/; (Hruz et al., 2008), which aphids do not consume at all. This speculation is confirmed by our gene induction experiments, which shows that the expression of *SKS13* is not induced at all upon aphid infestation (Chapter 5). These results are in

agreement with microarray studies of aphid infested *A. thaliana*, none of which has identified these genes as candidates to be involved in plant defense (Moran et al., 2002; De Vos et al., 2005; Kempema et al., 2007). The identification of these two genes perfectly matched our hypothesis that some genes, once being (over)expressed in leaves may confer increased aphid resistance.

Another common feature of these two genes is that the different levels of expression do not determine the resistance level. In Chapter 4, I observed a hundred fold difference in the expression level of *IRM1* in mutant 3646 and transgenic plants, whereas the number of aphids on these *IRM1* overexpressing lines was comparable. Similar results were found for *SKS13* overexpressing lines, in which identical insect resistance levels were conferred by significantly different expression levels of *SKS13* in mutant 3790 and the transgenic plants (Chapter 5). This suggests that both genes are functioning in networks, i.e. once their transcript levels reach a certain threshold other factors become limiting.

Furthermore the increased insect resistance conferred by these two genes is not aphid species specific as is indicated by the fact that both the generalist *M. persicae* and the specialist *B. brassicae* are affected (Chapter 4 and 5). Previously reported aphid resistances are highly insect specific and only effective against one aphid species. Such insect species-specificity is normally reported for *R* gene mediated resistance. For instance, the melon *Vat* gene confers resistance against melon aphids (Kennedy et al., 1978) and the *Rag* gene identified in soybean confers resistance to soybean aphids (Crompton and Ode, 2010). However there are also some metabolite mediated resistances that show species-specificity. The *A. thaliana* wax mutant *cer3* (At5g02310) only reduces *B. brassicae* population development and has no effect on *M. persicae* (Rashotte, 1999). Glucosinolates have been shown to be a strong deterrent for *M. persicae* (Kim and Jander, 2007; Pfalz et al., 2009), but negative effects of glucosinolates on *B. brassicae* have not been reported. The non-aphid-species-specific resistance conferred by *IRM1* and *SKS13* may be very useful in agricultural applications.

The location of resistance factors and their effects on virus transmission

Although phloem feeding insects, such as aphids and whiteflies, increase their populations quickly on plants, the direct damages caused by phloem sap consumption are relatively limited compared to the indirect damages resulting from the transmitted viruses. Therefore, it is important to uncover plant resistance that affect insects and reduce virus transmission as well. Studying insect feeding behavior by the Electrical Penetration Graph (EPG) technique can provide clues about the location of resistance factors (Tjallingii, 1990; Tjallingii et al., 2010). By using EPG I characterized the resistance of mutant 435, 3646 and 3790, revealing different locations of resistance factors (Figure 1) and effects on the transmission of circulative virus.

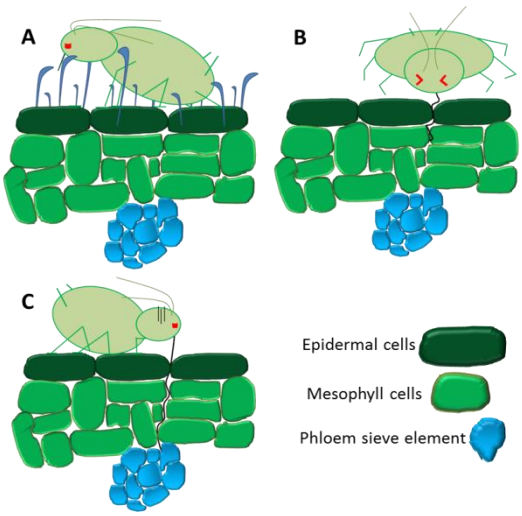

Figure 1. Aphid probing and feeding behavior affected by different locations of plant resistance factors. A. Surface resistance. Aphids are trapped by the sticky exudates of the trichomes on the surface of leaves (usually found in potato and tomato but not in *Arabidopsis thaliana*). B. Epidermal/mesophyll resistance. Aphids encounter penetration difficulties during stylet pathway phase (mutant 3646). C. Phloem-based resistance. The phloem sap may not be favored (mutant 435) or even toxic (mutant 3790) to aphids.

Epidermis/mesophyll resistance

The EPG analysis revealed that on one of the mutants (number 3646) the aphids encountered resistance when reaching the phloem (Chapter 4). I speculate that a mechanical barrier may be behind this. I am tempted to associate the mechanical barrier to cell wall metabolism based on the fact that in wild type plants the expression of the responsible gene *IMR1* is strong in xylem in which the plant cells are highly lignified and rigid (Northcote, 1989). Gene *IMR1* had not been characterized before and very limited information is available to further speculate the role of *IRM1* in aphid resistance in plants. Future experiments on the protein encoded by the *IRM1* gene, e.g. subcellular localization as well as its activity in plants and aphids, will help to provide insight into the function of *IRM1*.

Phloem-based resistance

The other two mutants (435 and 3790) display phloem-based resistance, which supports the observation that most of the reported plant resistance mechanisms to phloem feeding insects are phloem based. Phloem is the place where the insects feed and a likely place for plants to activate a defense response. Callose deposition and forisome dispersion leads to sieve element occlusion, which blocks the food canal of the insects (Will et al., 2007; Hao et al., 2008). Furthermore, detrimental factors are found in the phloem sap of an *MPL1* (*MYZUS PERSICAE-INDUCED LIPASE* 1) overexpressing plant and in the *ssi2* mutant (Louis et al., 2010). Most phloem-based resistances lead to a reduction in the time spent by aphids on phloem feeding. For example, the time spent by aphids on phloem feeding was longer on susceptible mutants *pad4* and *tps11,* but shorter on resistant transgenic plants overexpressing *PAD4* when compared to wild type plants (Pegadaraju et al., 2007; Singh et al., 2011).

Aphids on mutant 435 more frequently start phloem salivation and phloem sap ingestions than aphids on the wild type. Also the mean duration of the phloem sap ingestion was shorter, whereas the total time of the phloem sap ingestion between mutant and wild type did not differ (Chapter 2). I did not manage to obtain the information about the affected gene that could have provided clues about the mechanism. However, based on the aphid feeding pattern I can exclude the possibility of callose deposition and forisome dispersion being involved in this mutant (Will et al., 2007; Hao et al., 2008). The feeding pattern of the aphid on the mutant showed similarities to mutants that produce excess antifeedant compounds (Mndolwa et al., 1984; Kim et al., 2008), but so far I could not identify any reported compounds that may explain the requirement for intact plants to express the aphid resistance. Compared to mutant 435, aphid resistance on mutant 3790 is also phloem-based but stronger, which is indicated by a significant reduction in the total time of phloem feeding of aphids. In addition aphids on mutant 3790 showed a prolonged time to the start of the sustained phloem ingestion (E2) phase, suggesting that the resistance of the mutant becomes effective once the aphid makes contact with the phloem (Chapter 5). The stronger phloem-based resistance of mutant 3790 than mutant 435 is further supported by the fact that mutant 3790 shows an effect on aphid performance in no-choice situations and mutant 435 does not. The resistance of mutant 3790 may be explained by the function of the responsible gene *SKS13*. Overexpression of *SKS13* in leaves generates excessive reactive oxygen species (ROS) (Chapter 5). ROS accumulation is a common response to pathogens, which could act as a direct defense or serve as signaling molecules to active downstream plant defense responses (Miller et al., 2009). Recently, several studies have suggested that ROS plays a role in plant resistance against insects (Maffei et al., 2007; Kerchev et al., 2012).

Insect resistance versus virus resistance

Mutant 3646 and 3790 have been identified in virus transmission assays, in which plants of these two mutant lines showed reduced virus transmission. The reasons of the reduced virus transmission are

revealed by the EPG analysis. Although the resistance factors of mutant 3646 and 3790 were in different locations, they both severely affected the sustained phloem sap ingestion of aphids. Less aphids show sustained phloem sap ingestion on these two mutants compared to wild type and the aphids that show this activity on the mutants did this less frequently (Chapter 4 and 5). Sustained phloem sap ingestion is required for the transmission of the circulative viruses (Hogenhout et al., 2008). As a result virus transmission is reduced on these two mutants. Sustained phloem sap ingestion is not affected in mutant 435 (Chapter 2), which is in agreement with the fact that this mutant has not been identified in virus transmission assays.

In addition to insect resistance genes, there are many virus resistance genes. The *N* gene of tobacco confers resistance to tobacco mosaic virus (TMV) and other tobamovirus family members (Whitham et al., 1996). The *Ty-1* to *Ty-5* genes have been shown to confer resistance to Tomato yellow leaf curl virus (TYLCV) (Michelson et al., 1994; Anbinder et al., 2009; Ji et al., 2009; González-Cabezuelo et al., 2012). The *Ty-1/Ty-3* gene was shown to be a completely new class of resistance genes (Verlaan et al., 2013). Some virus resistances reduce virus accumulation and symptoms of infected plants, but may breakdown under high virus pressure (Lapidot and Friedmann, 2002). In this case, insect resistance will reduce pressure on the virus resistance by decreasing the number of vector insects. On the other hand plants that are infected with virus may become more attractive to insects than healthy plants (Mauck et al., 2010). In this case virus resistance can in turn decrease the virus induced attraction of plants to vector insects Combination of both resistances will increase the durability of virus resistance in crop plants, a welcome addition to agriculture.

Perspectives for breeding insect resistant plants

Plant translational genomics

Nowadays with the development of high throughput gene characterization in model plants, such as *A. thaliana*, a new field of "plant translational genomics" has emerged. Through translational genomics knowledge obtained in model plants can quickly be applied in crops (Stacey and VandenBosch, 2005). One form of plant translational genomics is the candidate gene approach (Salentijn et al., 2007). This approach is based on the assumption that genes with a proven or predicted function in model plants could control similar functions in crop plants. The implementation has been well illustrated with several examples, although not for insect resistance. Following the identification of a stress responsive DREB/CBF type transcription factor in *A. thaliana*, five DREB homologues were isolated in rice. Increased tolerance to several abiotic stresses was observed in *A. thaliana* and rice that overexpressed the *A. thaliana DREB1A* gene (Ito et al., 2006; Sakuma et al., 2006). Transgenic *A. thaliana* overexpressing rice *OsDREB1* also resulted in increased tolerances, indicating that both orthologs are functionally conserved between *A. thaliana* and rice. A similar situation was shown to exist for genes involved in early pot shatter that leads to severe seed yield losses in *Brassica* species. Several candidate genes for this trait have been identified in *A. thaliana*, *INDEHISCENT* (*IND1*) being one of them (Liljegren et al., 2004). Two *IND1* orthologs isolated from *Brassica* species were able to complement the *A. thaliana ind1* mutant phenotype, demonstrating that these two *Brassica* genes have the same functions as *A. thaliana* gene *IND1*.

The *A. thaliana* genes *IRM1* and *SKS13* that confer resistance to phloem feeding insects once being overexpressed in *A. thaliana* plants (Chapter 4 and 5) are also good candidates for a translational genomics approach. Many crop plants, such as rice and tomato suffer a lot from the damages caused by phloem feeding insects. Identification of the orthologs are facilitated by the availability of the whole genome sequences of rice (Goff et al., 2002; Yu et al., 2002) and tomato (Consortium, 2012).

Application of the findings can be through either expression of the gene identified in the model species in the crop plant or by searching for genetic variation for the homologues in the crop plant itself. The identification of other insect resistant *A. thaliana* mutants (Chapter 2 and 3) offers even more possibilities.

In addition to using the genes already identified, other genes that are included in the same gene family or involved in the same biological functions as the identified genes may also be considered as candidate genes for further investigation. In case of *SKS13* (Chapter 5), other members in this family encode multicopper oxidase-like proteins as well, sharing highly conserved copper-binding sites (Lamesch et al., 2012). Interestingly, eight members display identical expression profiles as *SKS13* and would also be promising candidate genes to further characterize their roles in insect resistance. I also suggested that the insect resistance conferred by *SKS13* is likely due to the ROS accumulation in leaves (Chapter 5). The role of ROS in insect resistance has been shown in several studies; however, only one gene, *RBOHD* that encodes an enzyme promoting ROS accumulation, has been demonstrated to be involved in insect resistance. ROS scavengers also affect plant resistance to insects, as indicated by an increased level of ascorbate acid, a compound reducing ROS accumulation, that decreased the plant resistance to insects (Kerchev et al., 2012). It is reasonable to propose that genes involved in ROS generating and scavenging can also be considered as candidate genes to be involved in insect resistance. Moreover, the effect of *IRM1* overexpressing plants on aphid feeding behavior suggests that cell wall reinforcement may be involved in insect resistance. In this sense, transcription factors *MYB75* and *SND2* that regulate secondary cell wall deposition in *A. thaliana* (Bhargava et al., 2010; Hussey et al., 2011) may become candidates to verify their functions in insect resistance.

Trade-offs resulting from increased resistance

It should be kept in mind that the plant resistance to (a)biotic stresses may be costly (Heil and Baldwin, 2002; Kempel et al., 2011). As indicated by our results, the increased insect resistance was accompanied by a reduced plant growth fitness for all *A. thaliana* mutants with increased insect resistance (Chapter 2, 3, 4 and 5). A growth retardation has also been reported in *OsDREB1A* overexpressing transgenic plants that display tolerance to drought, high-salt and cold stress (Dubouzet et al., 2003). Although resistance is often accompanied with a growth reduction, it is not always the case and even unpredictable. The *A. thaliana* plants overexpressing *HARDY* gene display smaller and thicker leaves than the wild type (Karaba et al., 2007). However, transgenic rice plants overexpressing the *A. thaliana HARDY* gene showed an enhanced resistance to drought under stress conditions and were able to grow much better than wild type under no-stress conditions (Karaba et al., 2007). Further investigations of transgenic crop plants overexpressing *IRM1* and *SKS13* as well as genes that still have to be identified from other insect resistant mutants (Chapter 2 and 3) will reveal the effects of these genes in crops.

It has been suggested that genes under control of the CaMV 35S promoter, that are constitutively expressed in all plant tissues, are likely to result in growth penalties because the plant uses more resources than necessary for their defense. If this is the case, this problem can be solved by using specific promoters. For instance, the stress-inducible *rd29A* promoter regulates the overexpression of *DREB1A* in transgenic tobacco and wheat, which minimized the adverse effects on plant growth (Kasuga et al., 1999; Pellegrineschi et al., 2004). Tissue specific promoters can also help to reduce the unnecessary costs. Trichome-specific promoters can be used to produce active compounds, such as zingiberene, resins and terpenoid lactones that act as direct toxins to insects (Szczepanik et al., 2005; Bleeker et al., 2012). Genes under control of phloem-specific promoters can also provide resistance to phloem feeding insects (Shi et al., 1994).

Other strategies to improve insect resistance in crops

Once the genes involved in insect resistance have been identified, there are several strategies to apply them into improvement of crop resistance. The successes of insect resistant transgenic plants have been demonstrated by many examples. Transgenic crops that express bacterium *Bacillus thuringiensis* (*Bt*) effectively suppress the lepidopteran and coleopteran (leaf-chewing) insects (Gatehouse, 2008) (Sanahuja et al., 2011). Transgenic rice plants that express the snowdrop lectin showed resistance to brown planthopper and other phloem feeding insects (Foissac et al., 2000). Expression of garlic lectin in transgenic rice not only conferred resistance to phloem feeding insects but also decreased the transmission of viruses vectored by the insects (Saha et al., 2006). Recently, virus-induced gene silencing is used to manipulate metabolic pathways to improve insect resistance in crops (Besser et al., 2009; Schilmiller et al., 2012). For instance, two forms of diterpenes are produced in one metabolic pathway in tobacco, one form is toxic to insects and the other one is not. In cultivated tobacco, via the virus-induced gene silencing, the production of the non-toxic form of diterpenes is reduced and in turn the toxic form of diterpenes is promoted (Wang et al., 2001). A new promising alternative is the use of RNAi to silence essential genes in the pest insects (Mao et al., 2007; Huvenne and Smagghe, 2010; Zha et al., 2011; Xue et al., 2012; Burand and Hunter, 2013; Gu and Knipple, 2013). This can be accomplished by the production of dsRNA molecules, either in the host plant (Baum et al., 2007) (Pitino et al., 2011) or by production of dsRNA in the lab and using it as a highly specific insecticide (Wang et al., 2011; Zhang et al., 2013). TILLING, a technique to select induced mutations in targeted genes is an alternative approach directly useable to evaluate the effects of mutation on insect resistance in crop plants (Slade et al., 2005). However, this approach is limited by the number of candidate genes and the further breeding process that is required to combine mutated genes and to purge background mutations.

Concluding remarks

Development of high-throughput screening methods helps the identification of *A. thaliana* mutants that show increased resistance towards aphids. The subsequent characterization of the mutants revealed two genes conferring enhanced aphid resistance via different mechanisms. These findings contribute to a better insight into the interactions between *A. thaliana* and phloem feeding insects at the molecular level. The next step is to transfer the knowledge obtained in *A. thaliana* into crop plants. This can be achieved by developing transgenic crop plants that express the gene identified in *A. thaliana* or by developing markers based on the genetic variation of the homologues genes in the crop plant itself. Newly developed techniques, such as RNAi and TILLING should facilitate the transgenic and genetic studies of insect resistance in crops, as well.

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Summary

Phloem feeding insects are among the most devastating pests worldwide. They not only cause damage by feeding from the phloem, but also by vectoring plant viruses. During their evolution plants have developed a variety of defense traits to combat insects. These plant resistance traits can be antixenotic and/or antibiotic. Antixenosis is the first line of defense that prevents insects from landing and settling, while antibiosis reduces the population development of the colonizing insects. In this project we aimed at identifying genes that can increase resistance towards phloem feeding insects and also prevent, as far as possible, transmission of viruses. Acknowledging that changing the expression level or expression localization of genes might increase resistance, we screened an *Arabidopsis thaliana* activation tag gain-of-function mutant collection for increased resistance towards the green peach aphid (*Myzus persicae*). In these mutants, tagged genes are overexpressed by the strong 35S enhancer adjacent to the natural promoter that results in a dominant gain-of-function phenotype. The overexpression of a particular gene in such mutants may result in enhanced resistance to aphids and other phloem feeding insects.

To identify mutants with increased insect resistance efficient and reproducible screening methods needed to be developed first. Based on the hypothesis that there is a trade-off between plant fitness and plant resistance, we first screened a subset of 170 mutants that were previously selected based on their reduced growth to increase the chance of identifying mutants with increased resistance. In this screening we used choice assays and selected one mutant that displays enhanced antixenosis based resistance towards aphids. Further characterization of this mutant revealed that that the antixenosis is phloem based and requires intact plants.

To evaluate aphid resistance of a larger number (>5000) of activation tag mutants, we established a high throughput screening system in which plant resistance against aphids is inferred from a reduced transmission of the circulative *Turnip yellows virus* (TuYV). This virus can only be transmitted into a plant after virus-infected aphids feed for a prolonged (> 10min) time from the phloem sap. In the initial screening 13 virus-free mutant lines were identified. The putative candidate mutant lines were re-evaluated and characterized, resulting in nine mutants on which aphids showed a reduced population development.

Molecular analysis of two of these mutants revealed that the genes underlying the resistance were *IRM1* (*Increased Resistance* to *Myzus persicae 1*, At5g65040) and *SKS13* (*SKU5 Similar 13*, At3g13400). In wild type plants, *IRM1* is strongly expressed in xylem and extremely low expressed in other plant tissue whereas *SKS13* is exclusively expressed in pollen. We show that constitutive overexpression of these genes in all plant tissues confers enhanced resistance towards aphids. Analysis of aphid feeding behavior showed that the resistance conferred by *IRM1* and *SKS13* affect the aphids differently. On the *IRM1* overexpressing mutant aphids encounter difficulties in reaching the phloem, indicating that resistance factors are located between the cell surface and the phloem. On the *SKS13* overexpressing mutant the phloem feeding of aphids is severely affected, indicating that resistance factors are phloem based. Further analysis strongly suggests the involvement of Reactive Oxygen Species (ROS) in the reduced aphid performance on the *SKS13* overexpressing mutant. We also show that the resistances are not aphid specific, as the performance of the cabbage aphid (*Brevicoryne brassicae*) is also affected on both overexpressing mutants.

The results obtained in this thesis show that plant resistance to insects can be increased by expressing genes that are assigned for other biological functions. Characterization of the identified mutants revealed two genes conferring enhanced aphid resistance via different mechanisms. These findings lead to a better understanding of plant-aphid interactions on the molecular level. Furthermore, such knowledge obtained from the model plant *A. thaliana* should be applied in crop plants, which can be achieved by transgenic and genetic studies in combination with newly developed techniques, such as RNAi and TILLING.

Samenvatting

Wereldwijd behoren insecten die zich voeden met floëemsap (ook wel zuigende insecten genoemd) tot de meest schadelijke voor onze gewassen. Ze veroorzaken niet alleen schade door het voeden, maar ook via de virussen die ze overdragen. Gedurende de evolutie hebben planten verschillende mechanismen ontwikkeld om zich tegen insecten te verdedigen. Deze resistentie eigenschap van de plant kan gebaseerd zijn op antixenose en/of antibiose. Antixenose is de eerste verdedigingslinie die insecten verhindert op de plant te landen en zich daar te vestigen. Antibiose vertraagt de populatie ontwikkeling van de koloniserende insecten. In dit project hebben we geprobeerd om genen te identificeren die de resistentie van planten tegen zuigende insecten kan laten toenemen en voor zover mogelijk het overbrengen van virussen verhinderen. We gaan er daarbij van uit dat deze genen al in de plant aanwezig zijn en dat we de resistentie kunnen verhogen door het veranderen van hun expressie niveau of de plaats waar ze tot expressie komen.

Om dergelijke genen te vinden hebben we een zgn. *Arabidopsis thaliana* activation-tag mutanten collectie gescreend op verhoogde resistentie tegen de groene perzikluis (*Myzus persicae*). In dergelijke mutanten worden genen tot overexpressie gebracht met behulp van een sterke 35S promotor die via een transposon in het genoom van *A. thaliana* is ingebracht. Dit resulteert in een dominant 'gain-of-function' fenotype. De overexpressie van sommige genen in deze mutanten kan leiden tot verhoogde resistentie tegen bladluizen en andere zuigende insecten.

Om mutanten te identificeren met verhoogde resistentie tegen insecten zijn efficiënte en reproduceerbare screeningsmethoden nodig welke eerst ontwikkeld moesten worden. Gebaseerd op de hypothese dat er een afweging is tussen plant productiviteit en resistentie, hebben we eerst een relatief kleine set van 170 mutanten onderzocht die geselecteerd waren op verminderde groei. Hiermee hoopten we de kans op het identificeren van mutanten met verhoogde resistentie te verhogen. Voor dit deel van het onderzoek hebben we gebruik gemaakt van keuze toetsen en een mutant geselecteerd die een versterkte antixenose gebaseerde resistentie tegen bladluizen liet zien. Uit de verdere karakterisering van deze mutant bleek dat de antixenosis floëem gebaseerd was en intacte planten vereiste.

Om bladluisresistentie in een groter aantal (> 5000) activation-tag mutanten te identificeren, hebben we een zogenaamd high throughput screening systeem ontwikkeld, waarin de resistentie tegen bladluizen wordt afgeleid uit een verminderde transmissie van het circulative *Turnip yellows virus* (TuYV). Dit virus kan alleen op een gezonde plant worden overgedragen via virus besmette bladluizen indien ze gedurende een langere tijd (> 10min) kunnen voeden van het floëem sap. In een eerste screening met ongeveer 5000 mutanten werden er 13 mutanten gevonden die vrij van virus waren gebleven. Deze kandidaten zijn in detail gekarakteriseerd, wat resulteerde in negen mutanten waarop bladluizen een verminderde populatieontwikkeling lieten zien.

Moleculaire analyse van twee van deze mutanten liet zien dat de genen die verantwoordelijk waren voor de verhoogde resistentie *IRM1* (*Increased Resistance to Myzus persicae 1*; At5g65040) en *SKS13* (*SKU5 similar 13*; At3g13400) waren. In wildtype planten komt IRM1 sterk tot expressie in het xyleem en vrijwel niet in andere plantenweefsel terwijl SKS13 uitsluitend in pollen tot expressie komt. We konden laten zien dat, als deze genen constitutief tot (over)expressie werden gebracht in alle weefsels van de plant, dit leidde tot een verhoogde resistentie tegen bladluizen. Uit de analyse van het voedingsgedrag van de bladluis bleek dat de door *IRM1* en *SKS13* veroorzaakte resistentie verschilde

voor de twee genen. Op de *IRM1* overexpressie lijn ondervinden bladluizen moeilijkheden bij het bereiken van het floëem, wat duidt op resistentiefactoren die zich tussen het celoppervlak en het floëem bevinden. Op de *SKS13* overexpressie lijn is het voeden van het floëem sterk beïnvloed, wat aangeeft dat de resistentiefactoren floëem gebaseerd zijn. Nadere analyse wijst er sterk op dat de vorming van de zogenoemde reactive oxygen species (ROS) een rol speelt bij de verminderde prestaties van de bladluis op de *SKS13* overexpressie lijn. We tonen ook aan dat de resistentie niet bladluis specifiek is; ook de melige koolluis (*Brevicoryne brassicae*) ontwikkelt zich slechter op beide overexpressie lijnen.

De in dit proefschrift beschreven resultaten tonen aan dat de resistentie van planten tegen zuigende insecten kan worden verhoogd door expressie van genen, die een rol spelen in andere biologische processen, te verhogen. Karakterisering van twee van de geïdentificeerde mutanten liet zien dat de verhoogde luisresistentie in deze mutanten via verschillende mechanismen werd bewerkstelligd. Deze resultaten leiden tot een beter begrip van de plant-bladluis interactie op moleculair niveau. Verder kan de kennis verkregen in de modelplant *Arabidopsis* mogelijk toegepast worden om bladluisresistentie in cultuurgewassen te verkrijgen/verbeteren via transgene of genetische benaderingen al dan niet in combinatie met nieuw ontwikkelde technieken, zoals RNAi en TILLING.

中文摘要

刺吸式口器昆虫是全球最具破坏性的害虫。它们不仅通过刺吸式口器取食植物韧皮部汁液危害植物，而且传播植物病毒。在进化中，植物产生了各种防御特征来抵御昆虫。这些防御特征可表现为抗选择性和抗生性。抗选择性阻止昆虫着陆安顿在植物上，是植物的第一道防线；而抗生性减缓已安顿在植物上的昆虫种群数量的增长。在这个项目中，我们的目标是鉴定出能够增强植物对刺吸式口器昆虫抗性的基因从而尽可能的减少植物病毒的传播。改变基因表达水平或位置可能会增强植物的抗虫性。基于此我们在一个拟南芥的激发标签功能获得突变体库中筛选对桃蚜有增强抗性的突变体。在这些突变体中，标签基因由于该基因启动子临近35S增强子而过表达，从而产生获得功能的表型。在这些突变体中由于某些基因的过表达植物可能表现出对蚜虫以及抗其他刺吸式口器昆虫增强抗性。

要发现增强了抗虫性的突变体首先需要开发高效可重复的筛选方法。我们先筛选了170个在之前研究中挑选出来的有减缓生长的表型的突变体。基于植物生长适合度和植物抗性之间的交易假设，筛选这些突变体可能会提高发现抗虫表型的机会。在筛选中我们使用选择性试验，发现了一个突变体表现出对蚜虫的抗选择性。进一步的鉴定显示了这个突变株对蚜虫的抗选择性位于韧皮部，且要求完整的植株。

为了更大规模地评估激发标签突变体对蚜虫的抗性，我们建立的一个高通量的筛选体系。在这个体系中植物对蚜虫增强的抗性由降低了的芜菁黄化病毒的传播来指示。这种病毒只有在感染了病毒的蚜虫对韧皮部汁液进行长于10分钟的取食后才会被传入被取食的植株。在对大于5000个突变体的初筛中我们发现了13个没有被病毒感染。在对这些候选的抗蚜虫突变体的二次评估和鉴定中，九个突变体确实降低了蚜虫种群数量的增长。

我们对于九个突变体中的两个进行分子分析，发现增强了的对蚜虫的抗性是基于基因*IRM1*和*SKS13*。在野生型植物中，*IRM1*在木质部导管中高表达在其他植物组织中表达量极低，而*SKS13*只在花粉中表达。我们发现在植物所有组织中持续过表达这两个基因能增强植物对蚜虫的抗性。蚜虫的取食行为分析表明*IRM1*和*SKS13*产生的抗虫性对蚜虫的影响是不同的。在*IRM1*过表达突变体中蚜虫的刺吸式口器在达到韧皮部的过程中遇到阻碍，表明抗虫因素位于表皮细胞和韧皮部之间。而在*SKS13*过表达突变体中蚜虫在韧皮部的取食受到严重的影响，表明抗虫因素位于韧皮部筛管内。进一步的分析暗示在*SKS13*过表达突变体中蚜虫表现的降低很可能是因为活性氧簇的参与。我们还发现由*IRM1*和*SKS13*持续过表达增强的抗虫性不具有蚜虫种的特异性，因为甘蓝蚜的表现在这两个过表达突变体上也受到负面影响。

这篇论文中的结果表明植物对昆虫的抗性可以通过增强基因的表达来实现，而这些基因原可能不是参与防御昆虫而是参与其它生物学功能的。对于两个突变株的鉴定揭示了两个基因通过不同的机制增强了植物对蚜虫的抗性。这些发现能够让我们在分子水平更好地了解植物和蚜虫的相互作用。此外，在模式植物拟南芥上获得的知识应通过转基因和遗传学研究手段并结合新技术，如RNA干扰和TILLING，尽可能的被应用到作物上。

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Curriculum vitae

Xi Chen was born on November 28th, 1983 in Jiangsu, China. She has obtained a bachelor in biological technology at Nanjing Normal University (Nanjing, China) in 2002. After graduation in 2006, she was enrolled in a PhD programme in Nanjing Agricultural University (Nanjing, China) under the supervision of Prof. Zhaojun Han. From 2009 to 2013, she conducted her PhD research in the laboratory of Plant Breeding at Wageningen University (Wageningen, the Netherlands). She worked on insect resistance in *Arabidopsis thaliana* under the supervision of Dr B.J. Vosman.

Publications

1. **Chen X**, Vosman B, Visser RG, van der Vlugt RA, Broekgaarden C (2012) High throughput phenotyping for aphid resistance in large plant collections. Plant Methods 8: 33-33
2. **Chen X**, Zhang Z, Visser RG, Broekgaarden C, Vosman B (2013) Overexpression of *IRM1* enhances resistance to aphids in *Arabidopsis thaliana*. PLoS One. 8, e70914
3. **Chen X**, Zhang Z, Visser RG, Vosman B, Broekgaarden C. Constitutive overexpression of the pollen specific gene *SKS13* in leaves enhances aphid resistance in *Arabidopsis thaliana* (submitted)
4. Robin P. Huibers, Dongli Gao, **Xi Chen**, Annelies E.H.M. Loonen, Richard G.F. Visser and Yuling Bai. ATHB13 overexpression links leaf morphology with pathogen resistance (in preparation)

Education Statement of the Graduate School Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Xi Chen
Date: 20 November 2013
Group: Plant Breeding, Wageningen University & Research Centre

1) Start-up phase		date
►	**First presentation of your project**	
	Identification and characterization of genes that increase resistance to insects	Jan 26, 2010
►	**Writing or rewriting a project proposal**	
	Identification and characterization of genes that increase resistance to insects	Jan 10, 2009
►	**Writing a review or bookchapter**	
►	**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, Utrecht University	Jun 01, 2010
	EPS PhD student day, Wageningen University	May 20, 2011
	EPS PhD student day, University of Amsterdam	Nov 30, 2012
►	**EPS Theme Symposia**	
	Theme 2 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 15, 2010
	Theme 3 'Metabolism and Adaptation', Wageningen UNiversity	Feb 10, 2011
	Theme 2 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 03, 2011
	Theme 2 'Interactions between Plants and Biotic Agents', Wageningen University	Feb 10, 2012
	Theme 2 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 24, 2013
►	**NWO Lunteren days and other National Platforms**	
	NWO-ALW meeting 'Experimental Plant Sciences, Lunteren	Apr 19-20, 2010
	NWO-ALW meeting 'Experimental Plant Sciences, Lunteren	Apr 04-05, 2011
	NWO-ALW meeting 'Experimental Plant Sciences, Lunteren	Apr 02-03, 2012
►	**Seminars (series), workshops and symposia**	
	Plant Sciences Seminars by Marcel Dicke 'Insect ecology: of plants and man' and Marcel Janson 'Physics, chemistry, and self-organization in cells'	Jan 12, 2010
	Plant Breeding Research Day 2010	Feb 08,2010
	Seminar by BGI: Genomics in China	Apr 01, 2010
	Plant Sciences seminars by Louise Vet (Entomolgoy) and Just Vlak (Virology)	May 10, 2010
	Workshop 'Technology transfer in the plant sciences'	May 20-21, 2010
	Seminar by Anne Endler 'Dissecting Cellulose Production in Arabidopsis'	Jun 15, 2010
	Plant Sciences seminars by Piet Boonekamp and Robert Chakovski 'creating the tools for plant health'	Oct 12, 2010
	Mini Symposium 'How to write a world-class paper'	Oct 26, 2010
	Seminar 'Plant-Insect Interactions: from Molecular Biology to Ecology'	Nov 11, 2010
	Seminar by Ian Henderson, (Unversity of Cambridge, School of the Biological Sciences Department of Plant Sciences 'Genetics and epigenetics'	Dec 13, 2010
	Mini-workshop ' How to catch a rat'	Dec 13, 2010
	Plant Sciences seminars by Andries Koops: 'Bioscience strategy on plant-based raw materials for a biobased economy' and Luisa Trindade: 'Targeted breeding for a Biobased Economy'	Dec 14, 2010
	Plant Sciences seminars by Jaap Molenaar (Biometris) 'Systems Biology, a flourishing issue' and Richard Immink (Bioscience, Plant Development Systems) 'From ABC to XYZ in flower development	Jan 01, 2011
	Plant Science semina, Rien van der Mas 'Development of sustainable farming systems: examples of cooperation between strategic and applied reseach' and Jan van de Zande ' Development of 'sustainable' spray techniques in fruit orchards'	Mar 07, 2011
	Plant Breeding Research Day 2011	Mar 08, 2011
	Plant Breeding in the Genomics Era	Nov 25, 2011
	Plant Breeding Research Day 2012	Feb 29, 2012
	Seminar by Sir David C. Baulcombe, ' Plant versus virus: defense, counter defense and counter counter defense'	Oct 10, 2012
►	**Seminar plus**	
►	**International symposia and congresses**	
	Conference Next Generation Plant Breeding (Ede, The Netherlands)	Nov 12-13, 2012
	IOBC-WPRS meeting (Avignon, France)	Jun 10-13, 2013
►	**Presentations**	
	NWO-ALW meeting 'Experimental Plant Sciences, Lunteren (poster)	Apr 04, 2011
	Theme 2 'Interactions between Plants and Biotic Agents' Utrecht (oral)	Jan 24, 2013
	IOBC-WPRS meeting (Avignon, Franch) (oral)	Jun 12, 2013
►	**IAB interview**	
►	**Excursions**	
	visit KEYGENE breeding company	Jan 26, 2012
	Subtotal Scientific Exposure	*11.2 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Postgraduate Course 'Bioinformatics: a User's Approach'	Mar 15-19, 2010
	Postgraduate Course 'Survival Analysis'	Jan 26-27, 2011
	Autumn School "Host-Microbe Interactomics'	Nov 01-03, 2011
►	**Journal club**	
	Literature Discussions "plant breeding"	2009-2013
►	**Individual research training**	
	Subtotal In-Depth Studies	*6.0 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Presentation skills	May 12-26, 2010
	Project- and Time Management	Nov-Dec, 2011
	Scientific writing	Apr 20-Jun 08, 2011
	Information Literacy, including Endnote	Apr 27-28, 2010
	Techniques for Writing and Presenting a Scientific Paper	Feb 15-18, 2011
	Reviewing a Scientific Paper	Dec 20, 2011
	Teaching and Supervising MSc Thesis students	2011-2012
	Mobilising your Scientific Network	Sep 18 and 25, 2012
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*7.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**32.5**

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 ECTS credits

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

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