The identification and characterization of genes controlling defense responses and resistance mechanism against *Myzus persicae* (green peach aphid) in *Arabidopsis thaliana*

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Abstract

Aphids are economically destructive insects, which deprive plants of photoassimilates. Applying chemicals and using natural enemies are the control strategies employed to combat aphids. Excessive usage of chemicals is not environmentally friendly, and could lead to the emergence of insecticide resistant aphid strains. On the other hand, using natural enemies has proven to be a slow method in combating aphid infestation. Owing to the disadvantages of these two strategies, we address in this study, host plant resistance as an alternative control measure for aphids. This project aims to identify genes controlling defense responses and resistance mechanisms against the green peach aphid (GPA), *Myzus persicae*, in the model plant *Arabidopsis thaliana*. Genes seen to be up- regulated at least two fold from two independent microarray studies from published results with emphasis on genes related to stress response were selected. These included; *Glutathione transferase 1* (*GST1*), *Early responsive to dehydration 5* (*ERD5*), and *Senescence associated gene 21* (*SAG21*). Activation tag lines with random *Zea Mays* En-1 transposons inserts that had previously demonstrated the potential of being aphid resistant were also included in this project. Additionally, the susceptibility genes *Mildew locus O (mlo), 2, 6, 12* were studied to investigate the role they play in plant-aphid interaction. We carried out non-choice and dual-choice tests to study the performance and preference *of M. persicae* on the activation tag lines as well as on knock-out mutants of the susceptibility genes and the genes selected from microarray studies. We found out that *M. persicae* preferred *sag21* mutants over wild-type plants in dual-choice test and concluded that *SAG21* is involved in antixenosis against *M. persicae.* Our study also showed that activation tag mutant line 3790 affects the preference and performance of *M. persicae*. From non-choice studies, we found *mlo2, and mlo6* mutants to negatively affect reproductive potential of *M.persicae* with *mlo2/mlo6/mlo12* mutant's resistance being age dependent. From qPCR results, there is significant induction of *SAG21* (Senescence marker gene) in infested *mlo-2* mutant and wild-type plants and similarly, *PAL1*, (salicylic acid marker gene) in infested *mlo-2* mutant. Senescence is associated with salicylic acid accumulation in *mlo* mutants' plant tissues. We therefore conclude that the resistance shown by the *mlo* mutants against GPA is as a result of premature leaf senescence.

Key words: *Arabidopsis thaliana, Myzus persicae,* Host plant resistance, induced plant defense, antixenosis, antibiosis, susceptibility genes, senescence, gene expression

Introduction

Insects contribute to crop damage leading to major yield reductions. Insects known to be notorious to plants include the phloem-sucking insects such as aphids and whiteflies. These insects are described as being economically destructive as they limit plants' productivity by manipulating the resource allocation patterns in the plant (Dixon, 1977) . Aphids being the largest group of phloem feeders cause direct damage to crops using their stylets, which they insert into phloem sieve elements to suck up phloem sap, which contains the photoassimilates. The end result is nutrients depletion which is manifested by necrosis, chlorosis and stunting of plants (Miles, 1999). Aphids also damage plants indirectly by being vectors of more than hundreds of plant viruses (Martin et al., 1997) as well as depositing honeydew on plants. Honeydew is a substrate for sooty mold growth that blocks light transmission to the leaf reducing the photosynthetic potential of a plant. This translates into slower growth and lower yields (Wood et al., 1988).

The economic impact is highly felt because of the short reproductive cycle of aphids and their clonal mode of reproduction, which results in a population increase within a short period of time (Dixon et al., 1993). Chemicals and biological approaches employing the use of natural enemies can control aphids. However, chemicals can destroy the ecosystem by being harmful to beneficial insects such as natural enemies and pollinators (Lewis et al., 1997). Additionally, aphids have also been seen to develop resistance to some pesticides (Devonshire and Field, 1991). Biological control by using natural enemies is slow in controlling large aphid populations(Kindlmann and Dixon, 1999). Both chemical and biological control could be enhanced when used in combination with host plant resistance(Kindlmann and Dixon, 1999). There are not many cultivated crops that are resistant to aphids(Broekgaarden et al., 2011).It is therefore imperative to develop resistant cultivars by exploiting host plant resistance.

Host plant resistance is mediated through two branches of plant immune system; PAMP-triggered immunity (PTI) and Effector-triggered immunity (ETI) (Jones and Dangl, 2006). During PTI, the plant recognises non-self-molecules such as elicitors from the aphids or self-molecules referred to as Damage molecular patterns (DAMPS) comprised of cell wall fragments from damage caused by stylet penetration (Miles, 1999).These then trigger a myriad of defense responses (Lotze et al., 2007).The defense responses result in systemic acquired resistance and induced resistance to insects (Karban et al., 1999). This form of resistance is not insect species specific and is dependent on systemic defenses that protect the plant during an attack and subsequent attacks by insects.

The other branch of plant immune system is ETI, and follows the gene for gene principle. Resistance (R) genes in the plant recognize specific elicitors introduced by aphids (Brotman et al., 2002) and switch on a defense response. The resulting insect species-specific type of resistance, is normally conferred by a single dominant gene, which is responsible for mediating resistance. Examples of R genes utilized for aphids control include; *Vat* gene in melon (Klingler et al., 2001), *Mi* gene in tomato (Rossi et al., 1998) and *Rag* genes in soybean (Li et al., 2007). Host plant resistance is dependent on plant defense response, which falls into two categories; direct and indirect type of response (Kusnierczyk et al., 2008). Direct defense involves a plant employing certain mechanisms/traits of resistance to protect itself from attackers by negatively influencing the attacker's preference (antixenosis) or performance (antibiosis). These traits can involve physical structures such as trichomes to restrict the movement of herbivores on plant surface (Lapointe and Tingey, 1984) or thicker cell walls to impede feeding(Mohase and Taiwe, 2010). The production of protease inhibitors and deterrent volatiles also protect plants from herbivore feeding. Additionally, Plants can also accumulate toxic secondary metabolites in their tissues such as glucosinolates (GS) (Harrewijn, 1993; Cole, 1994; Ciepiela and Sprawka, 2001; Tran et al., 1997; Tosh et al., 2001). Intact GS and its breakdown products, such as isothiocyanates and nitriles, affect the performance of generalists aphids such as *Myzus persicae*, and have also been demonstrated to limit aphid feeding(Kim and Jander, 2007). Aphid feeding has been shown to induce oxidative stress resulting in the production of ROS which could be toxic to the insect(Zhu-Salzman et al., 2004). Moreover, Plants accumulate reactive oxygen species (ROS) in response to biotic and abiotic stress. ROS has been associated with stress signaling which may result in systemic induction of genes involved in defense(Kerchev et al., 2012).Another defense strategy employed by plants is occlusion of phloem sieve plates. This limits nutrients availability to aphid. For instance, callose deposition on sieve plates has been observed after aphid feeding (Moran et al., 2002; Botha and Matsiliza, 2004; Will et al., 2007). Additionally, callose deposition has also been associated with resistance to *Aphis gossypii* in Melons having *Vat* gene (Villada et al., 2009). Moreover, *Glucan synthase-Like 5 (GSL5),* an important gene in callose metabolism, is also shown to be involved in Harpins, plant pathogenic bacteria proteins ($HrpN_{Ea}$) - induced *M.persicae* resistance (Zhang et al., 2011). Premature leaf senescence has been shown to be an effective direct defense strategy in Arabidopsis during *M. persicae* feeding. According to (Pegadaraju et al., 2005; Sappl et al., 2009), a plant can only support an aphid colony up to a certain density, beyond which it has to safeguard its resources. It could do this by redirecting flow of nutrients from heavily infested leaves to aphid free organs. This in turn leads to programmed cell death, which is crucial in

protecting nutrients that could have otherwise been utilized by aphids at the expense of the plant. Subsequently, the reproductive potential of aphids is also negatively affected due to the limited nutrition.

Both direct and indirect plant defenses can either be constitutively present i.e. already present in the absence of an attacker, or induced upon attack by herbivores. Constitutive defense could have a negative impact on plant growth and development as most of the energy is channeled to defense even when the plant is not in need of protection (Kerchev et al., 2012). Induced resistance on the other hand is only launched when a plant is under attack by directing the tailored gene products needed to protect the plant from further attacks (Karban et al., 1999). Signal transduction plays an integral role in induced defense by regulating the induction of defense genes. Key signaling hormones identified in these pathways include the phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Glazebrook, 2001). Cross talk between the different phytohormone pathways is important for a plant to fine tune its defense response to target the appropriate attacker (Verhage et al., 2010). It should also be noted that previous studies in many plants, including Arabidopsis, show that there exists a negative crosstalk between the JA and SA pathways (Kusnierczyk et al., 2007). Studies demonstrate the induction of JA-and SA-dependent signaling post aphid feeding (Moran et al., 2002; Kusnierczyk et al., 2007). This kind of signaling has been demonstrated to be important in plants‘ induced defense against aphids (Mewis et al., 2005; Pegadaraju et al., 2005; Gao et al., 2007). Despite there being several hormones involved, the outcome of an induced defense response based on hormone signaling molecules is dependent on the concentrations and composition of the blend of hormones produced (De Vos et al., 2005).

Induced responses in the plant sometime result in induced susceptibility. During induced susceptibility, susceptibility genes are expressed and these in turn dampen a plant’s defense response. Studies on susceptibility genes in plant-pathogen interactions have already been done(Nishimura et al., 2003; van Damme et al., 2009; Consonni et al., 2010). Knocking-out susceptibility genes has been shown to render plants resistant to a number of pathogens. However, undesired phenotypes may result from knocking-out some susceptibility genes. This then requires studying the mechanisms of functioning of these genes, and looking for ways of uncoupling the undesired phenotypes from the desired resistance conferred by the knock-out mutants. One susceptibility gene that has received a lot of attention is the *Mlo* gene. Recessive alleles (*mlo*) of *Mlo* locus in barley induced by mutation have been used for

decades to confer resistance to powdery mildew *Erysiphe graminis* f.sp. hordei (Buschges et al., 1997). Besides being recessive, this kind of resistance mediated by *mlo* alleles is monogenic, durable in the field and broad-spectrum to all isolates of *Erysiphe graminis.* The *Mlo* protein is hypothesized to be a negative regulator of leaf cell death and it deregulates the onset of multiple defense response (Buschges et al., 1997). Additionally, *Mlo* is referred to as a calmodulin binding protein which binds calcium; which is believed to initiate a systemic signaling cascade when its level in the phloem suddenly increases (Van Bel and Gaupels, 2004). In Arabidopsis, it has been demonstrated that *mlo-2* mediated powdery mildew resistance is dependent on aberrant accumulation of indolic metabolites in the mutant (Consonni *et al.*, 2010). Barley *mlo* mutants and Arabidopsis *mlo-2* mutants exhibit early senescence and spontaneous callose deposition during later developmental stages (Buschges et al., 1997; Consonni et al., 2010b). Although, orthologues of *Mlo* genes in *Solanum* sp. have been shown to be up-regulated two fold in response to *M. persicae* feeding (Alvarez., 2007). *mlo* mutants have not been studied in relation to *M. persicae* resistance.

To be able to fully utilize host plant resistance for the development of aphid resistant crops it is important to dissect these defense pathways and identify resistance and susceptibility genes involved. Molecular biological tools such as microarrays, knock-outs and activation tags have been employed in the identification of resistance/defense, and regulatory genes in many plant-insect interactions (Glazebrook, 2001; Jones, 2001). This study aims at identifying and characterizing genes controlling host plant resistance to *M. persicae* (green peach aphid-GPA) using the tools of the model plant *Arabidopsis thaliana*. In this study, we selected genes shown to be up-regulated at least two fold following GPA infestation in Arabidopsis. The selection was done based on two independent microarray studies, emphasis being placed on genes related to stress response (Wagner et al., 2002; Dixon et al., 2005; Kusnierczyk et al., 2008). Mutants of three *mlo* genes (Consonni et al., 2010), and five activation tag mutants (Chen et al., unpublished) were also incorporated in this project. The activation tag mutants contain the *Zea mays En-1* transposon inserts randomly in the Arabidopsis genome (Marsch-Martinez et al., 2002). Each of the mutants had previously been demonstrated to remain virus free even after being infested with virus infected aphids showing a possibility for resistance to GPA. The effect of the mutants on *M.persicae* preference and performance was investigated giving a lead to into the mechanism of resistance against GPA in Arabidopsis.

Methodology

Study system

- Plant: *Arabidopsis thaliana* mutant lines in which candidate genes have been knocked out by T-DNA insertions; *Arabidopsis thaliana* activation tag insertion lines(**Table** 1) .
- Aphid: *Myzus persicae* Sulzer (green peach aphid-GPA)

Table 1: candidate genes

Microarray studies Columbia (Col)	Susceptibility genes Columbia (Col)	Activation tag lines (Wassilewskija (Ws)
Glutathione transferase 1 (*GST1*)	Mildew locus O 2 (*MLO2*)	3790,1547
Early responsive dehydration 5 (*ERD5*)	Mildew locus O 6 (*MLO6*)	1540,2119
Senescence associated gene 21 (*SAG21*)	Mildew locus O 12 (*MLO12*)	2160

The T-DNA insertion lines were identified from the Salk collection and the seeds ordered from NASC. Seeds of *Arabidopsis thaliana mlo* mutants and activation tag insertion lines were obtained from plant breeding department at Wageningen University.

Plant growth and insect cultures

The plants were grown in a climate room receiving a photoperiod of 8L: 16D light/dark, temperature of 20°C±2 during the day, and 18°C±2 during the night, and relative humidity was maintained at 70%. *M. persicae* was reared on *Brassica rapa* (Chinese cabbages) in a controlled-environment growth room receiving photoperiod of 6L: 18D constantly maintained at 20-22°C. Apterous (wingless) aphids were used in this experiment.

Host plant selection

i) Dual-choice experiment

Three WT and three plants from one mutant line were placed in each arena (Figure 1). There were ten arenas, each arena representing a replicate in this experiment. The plants were organized in a systematic way in such a way that the mutants alternated with the WT plants in the arena. A water barrier separated the arenas from each other. Thirty aphid adults were placed in the middle of each arena and allowed to make a choice between WT and mutants. 24hrs later, the numbers of aphids per plant were counted and the total number of aphids on mutants/WT plants computed.

Figure 1: Dual choice experiment in arena

ii) Y-tube experiment

These experiments were conducted to find out if the preference of aphids for particular groups of plants in the arena was volatile dependent.The olfactory behaviour of the aphids was assessed with a Y-shaped glass tube olfactometer (Koschier et al., 2000) (Figure 2). Aphids were allowed to choose between odour-loaded air from three Arabidopsis plants from a particular mutant in one Y-tube arm and odour-loaded air from three WT Arabidopsis plants in the other Y-tube arm. Three, five-weeks-old plants were placed in each glass jar that was connected to one of the Y-tube arms. The length of the arms and base of the Y-tube was 5 cm, with an inner diameter of 0.5 cm. The arms of the y-tubes had sieves that prevented the aphids from reaching the plants. Air entering the set up was filtered through activated

charcoal into the glass jars and sucked into the y-tube. To ensure continuous good airflow through the Y-tube, air was sucked at the base of the Y-tube by a membrane pump maintaining airflow of 300mL/min. The set up was lit from above with artificial lighting to ensure equal distribution of light. An aphid was placed at the base of the Y-tube to make a choice within 7 minutes between the two Y-tube arms. A choice was considered to have been made when the aphid passed halfway beyond the junction of the two arms and not returning to the junction within 15 seconds. After testing six aphids, the odour sources were interchanged to avoid position effects and the plants were replaced with new ones after testing 12 insects. This experiment was conducted around noon for about three hours to reduce environmental variation, which could have an effect on the aphid behaviour. The experiment was terminated after testing 48 insects.

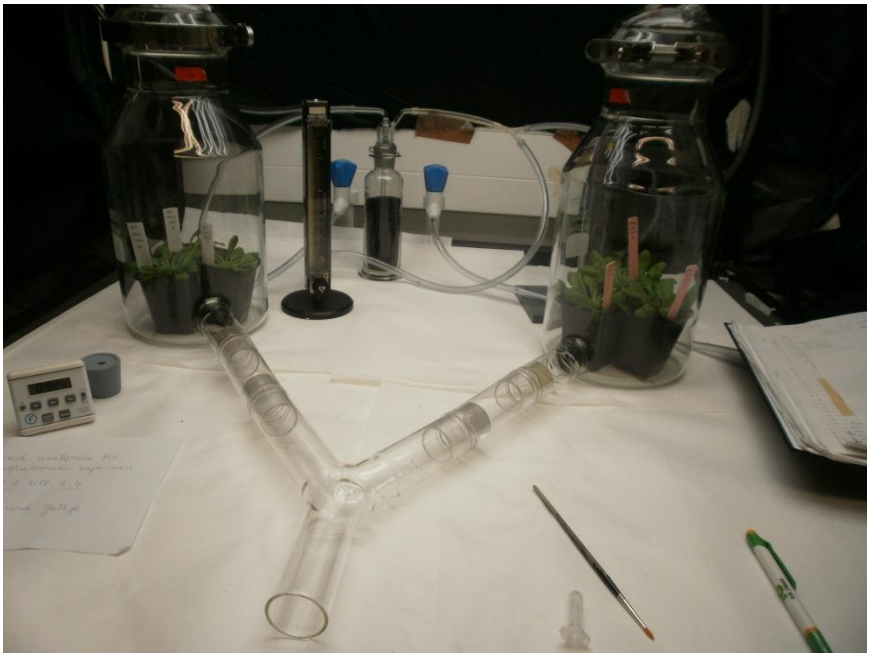


Figure 2: Experimental setup in Y-shaped glass tube olfactometer

Host plant suitability

The host plant suitability was determined in a non-choice test. An aphid culture made of adult aphids was synchronized overnight on a Chinese cabbage leaf in a petri dish. By using a fine paint brush, one day old aphid nymphs were then transferred to the adaxial side of a fully expanded leaf of a four weeks

old Arabidopsis plant. Each plant received one nymph. 20 wild- type (WT) plants and 20 mutants were used. To confine aphids on the individual plants, the infested plants were separated from each other by a water barrier. All the plants were placed in an insect proof netting cage. The experiment was set-up in a complete randomized design (CRD). The initially introduced nymphs were monitored every 24hrs to assess their survival and first day of reproduction. Finally, the total number of produced nymphs on each of the plants was monitored every 48hrs until 14 days after infestation. Nymph's survival/longevity, (Number of days the nymphs survived), pre-reproductive period (Number of days from nymph introduction to the first day of reproduction) and fecundity (mean number of newly borne nymphs within 14 days) were calculated.

Figure 3: Non-choice test experiment

Gene expression analysis

Quantitative real time-polymerase chain reaction (Q-RT-PCR) was used to study the transcript levels of callose metabolism genes, JA and SA markers, and senescence associated genes (SAGs) after aphid feeding. The target genes and their corresponding primer sequences are shown in (Table 2). This experiment had four treatments (1. Mutant infested, 2. WT infested, 3. Mutant uninfested, 4. WT uninfested) each having three biological replicates, and three plants pooled in each of the biological

replicates. For treatments 1 and 2, individual plants were infested with 50 apterous aphids of assorted ages. Fourty-eight hrs later, all samples were collected, freed from soil by brushing , and immediately frozen in liquid nitrogen. Aphids on the plants were gently brushed away and the uninfested plants were also brushed to simulate similar collection process. Total RNA was extracted by RNeasy Plant mini kit (Qiagen) preceding cDNA synthesis. RNA was quantified by Nano drop (ISOGEN-Life science). DNAse treatment was done to eliminate genomic DNA contamination prior to cDNA synthesis (Qiagen) on 1 µg of RNA. The iscript cDNA synthesis kit (BIO-RAD), was used for cDNA synthesis according to the manufacturer's instructions.

For qPCR, the cDNA was diluted 20 times. The Reaction mix constituted of; 2µl cDNA, 3 µl- 1µM gene specific forward and reverse primers, and 5 µl iQ SYBR green supermix (BIO-RAD). Actin 8 (At1g49240) was used as a reference gene to normalize gene expression across the samples to correct for differences in cDNA synthesis. qPCR was performed in a Real-Time thermal Cycler (Optical system software, version 2.0 for MylQ-BIO-RAD) following the cycles; 95°C for 3 min, then 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Melt curve analysis was performed to test the gene specificity of these primers. The gene fold change was analyzed using the formula: $2^{-\Delta ct}$ where 2 represents the doubling of RNA transcripts with each cycle with the assumption that PCR efficiency is 100% and -Δct is the difference in threshold cycles between the target gene and the reference gene (Livak and Schmittgen, 2001).

Data analysis

Data from dual choice test and odour experiment were analysed using χ^2 test with the null hypothesis that the aphids did not have a preference for one of the two plant groups/ odour sources. One-way ANOVA was used to analyze data from non-choice tests and gene expression. $Log_2(X)$ transformation was applied to gene expression data prior to analysis. All the data was analysed in genstat 14^{th} edition with confidence level of 95% applied.

Results

i). Host plant selection and host suitability of knock-out mutants from microarray studies

Dual -choice tests show aphids' preference for either mutants or WT plants. The aphids did not have a preference for either the *gst1* mutant or WT plants (χ2=0.05, d.f=1, p=0.830; Figure 4A). The same follows for the *erd5* mutant which also did not attract/repel aphids (χ^2=0.77, d.f=1, p=0.379; Figure 4A). On the contrary, the *sag21* mutant attracted more aphids compared to WT plants (χ^2=5.58, d.f=1, p=0.018; Figure 4A). To determine if this attraction was as a result of volatiles emitted by the *sag21* mutant, a Y-tube olfactometer experiment was conducted. From this experiment, 70% of the aphids did not make a choice between the *sag21* mutant and WT plants. While 18% made a choice for volatiles emitted by wild-type plants, 12% chose volatile emitted by the *sag21* mutant. Non-choice test was conducted to see if the mutants had an effect on aphids performance. From the non-choice test, there was no significant difference between the *gst1* mutant and WT plants in respect to pre-reproductive period (ANOVA, $F_{1,30}$=0.22, p=0.646); the pre-reproductive period was 8 days in both the *gst1* mutant and WT plants while the longevity (ANOVA, $F_{1,30}$=1.14, p=0.295) was 14 days in both *gst1* mutant and WT plants (Figure 4C). As for fecundity, there were 19% more nymphs born on *gst1* mutants compared to WT plants, but this was not a significant difference (ANOVA, $F_{1,30}$=2.39, p=0.133) (Figure 4B). Longevity and pre-reproductive period of aphids on *erd5* mutant were not significantly different from WT plants (ANOVA, $F_{1,23}$=0.06,P=0.813;$F_{1,23}$=0.09,P=0.767). Both the mutant and WT plants had a pre-reproductive period of 7.9 days. The mutant had longevity of 13.8 days while WT's longevity was 13.7 days (Figure 4C). The fecundity of aphids on *erd5* mutant was 11% lower compared to WT plants (Figure 4B), but again this was not significantly different (ANOVA, $F_{1,23}$=0.46, P=0.504). Aphids' performance in terms of longevity and pre-reproductive period was similar in *sag21* mutant and WT plants, both recording a pre-reproductive period and longevity of 7.8 and 14 days respectively (Figure 4C). The fecundity of aphids on the mutant was marginally higher than on WT plants by 6% (Figure 4B). However, this was not significantly different (ANOVA, F1, 38=0.79, P=0.378) from WT.

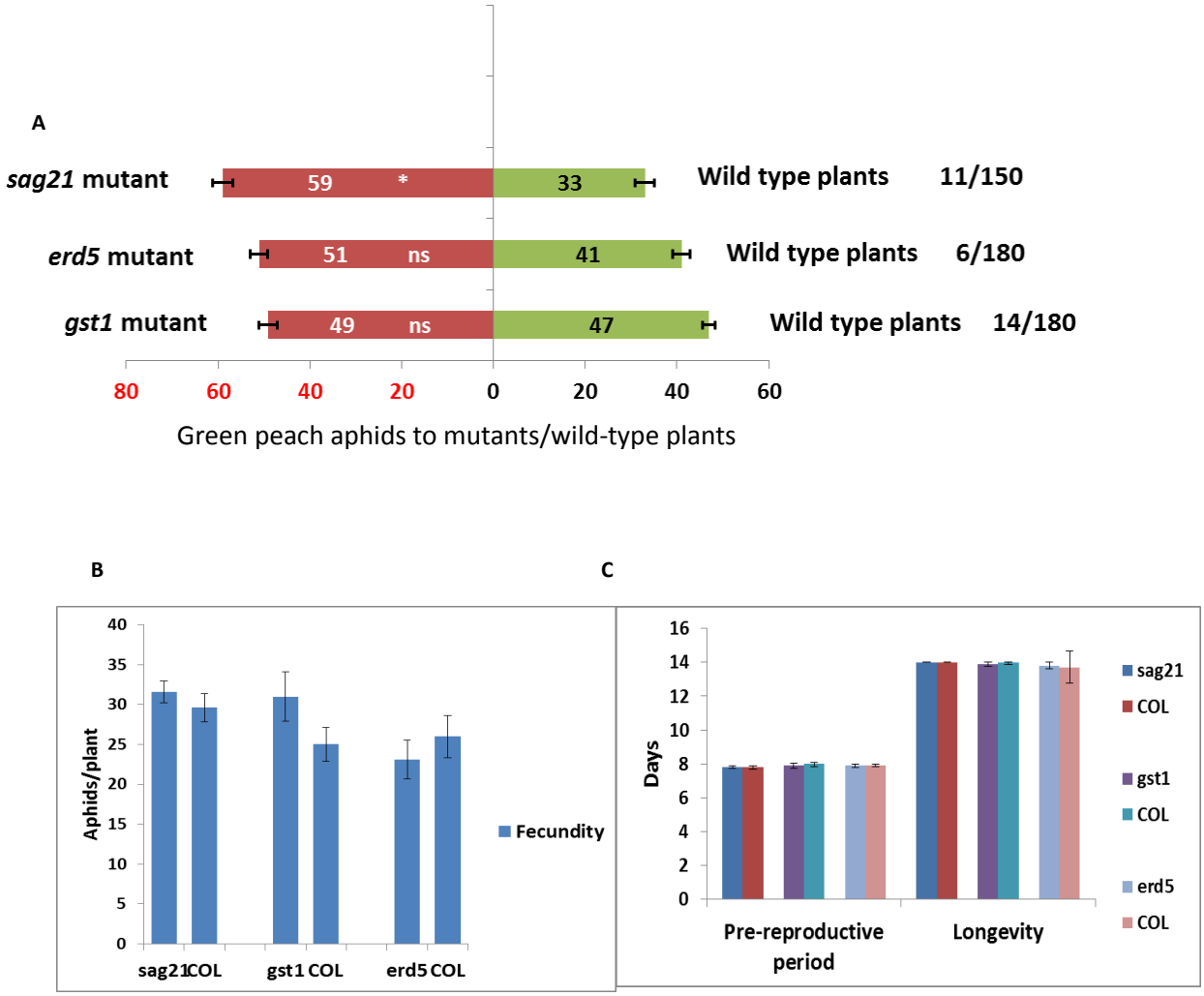


Figure 4: Host plant selection and performance of green peach aphid on the Arabidopsis mutants *sag21*, *erd5* and *gst1*.

A. **Host plant selection** :The number of aphids expressed in percentage (%) that made a choice for the mutants (red bars) and corresponding WT (green bars) in dual-choice test conducted in arena. Thirty apterous adult aphids were introduced in the middle of arena containing six 4 wks old plants(3 wild-type and 3 plants from one mutant). They were then allowed to make a choice within 24hrs. Total numbers of aphids on the mutants and wild-type plants were then counted. (Asterix(*) shows significance difference from WT while (ns) show no significance difference from WT at p≤0.05. The numbers of aphids that did not make a choice and the total numbers of aphids tested are shown at the far right of the figure. The bars represent SE±mean generated at a confidence level of 95%.

B. **GPA performance (Fecundity):**The number of nymphs borne by each of the introduced aphid on WT plants (Col) versus *gst1, sag21, erd5* mutants in a 14 day period in a non-choice test. The number of aphids was obtained by getting the average number of nymphs in the replicates used in each of the experiments. The columns represent SE±mean generated at a confidence level of 95%.

C. **GPA performance (Pre-reproductive period, Longevity):**The average number of days it took for the newly introduced nymphs to reach reproductive maturity (pre-reproductive period) on the mutants,*gst1, erd5,sag21* and WT plants (Col) and the survival of the nymphs within 14 days (longevity) on mutants and WT plants. The columns represent SE±mean generated at a confidence level of 95%.

ii). Host selection and host suitability on knock-out mutants of susceptibility genes

The triple mutant *mlo2/ mlo6/ mlo12* at four weeks recorded a pre-reproductive period of 8.1 days and longevity of 13.1 days. These two parameters were not significantly different (ANOVA,$F_{1,39}$=0,p=1; $F_{1,39}$=2.3,p=0.138; Figure 5A) from the WT. On the contrary, the mutant recorded significantly 25% lower aphid fecundity compared to the WT (ANOVA, $F_{1,39}$=8.85, p=0.005, Figure 5B). The mutant was even more resistant at 6 weeks and recorded an aphid fecundity and longevity of 82% and 77% lower than the WT, respectively. Moreover, this was seen to be significant different relative to WT (ANOVA, $F_{1,15}$=9.82, p=0.008;$F_{1,15}$=17.68,p<0.001; Figure 5). The nymphs introduced on the six weeks old mutants died after two days. The performance of green peach aphid on single and double mutants of *mlo2, mlo6, mlo12* genes was monitored to investigate the contribution of each of these genes to the resistance of the triple mutant. The *Pen3* mutant was included in this experiment to find out if the resistance shown by the triple mutant was independent of *PEN3* gene. Fecundity of aphids was monitored on the 14th day after their introduction on the WT and mutant plants. The aphids on WT plants showed a high fecundity of aphids as shown by the high number of aphid nymphs on the plants compared to the mutants. The *mlo2-11* and *mlo12* mutants had 12% less nymphs while the *mlo6/12* mutant had 4% less nymphs compared to the WT. Statistically, the fecundity of aphids on these mutants was not significantly different from WT (ANOVA,$F_{3,36}$=1.95,p=0.141). On the other hand, *mlo2-5, mlo2/pen3, pen3, mlo6, mlo2/6, mlo2/12* mutants had significantly lower numbers of aphid nymphs; (23%, 31%, 25%, 17%, 17%, 18% respectively) compared to WT plants (ANOVA,$F_{9,90}$ =3.73, p≤0.001;Figure 5C).

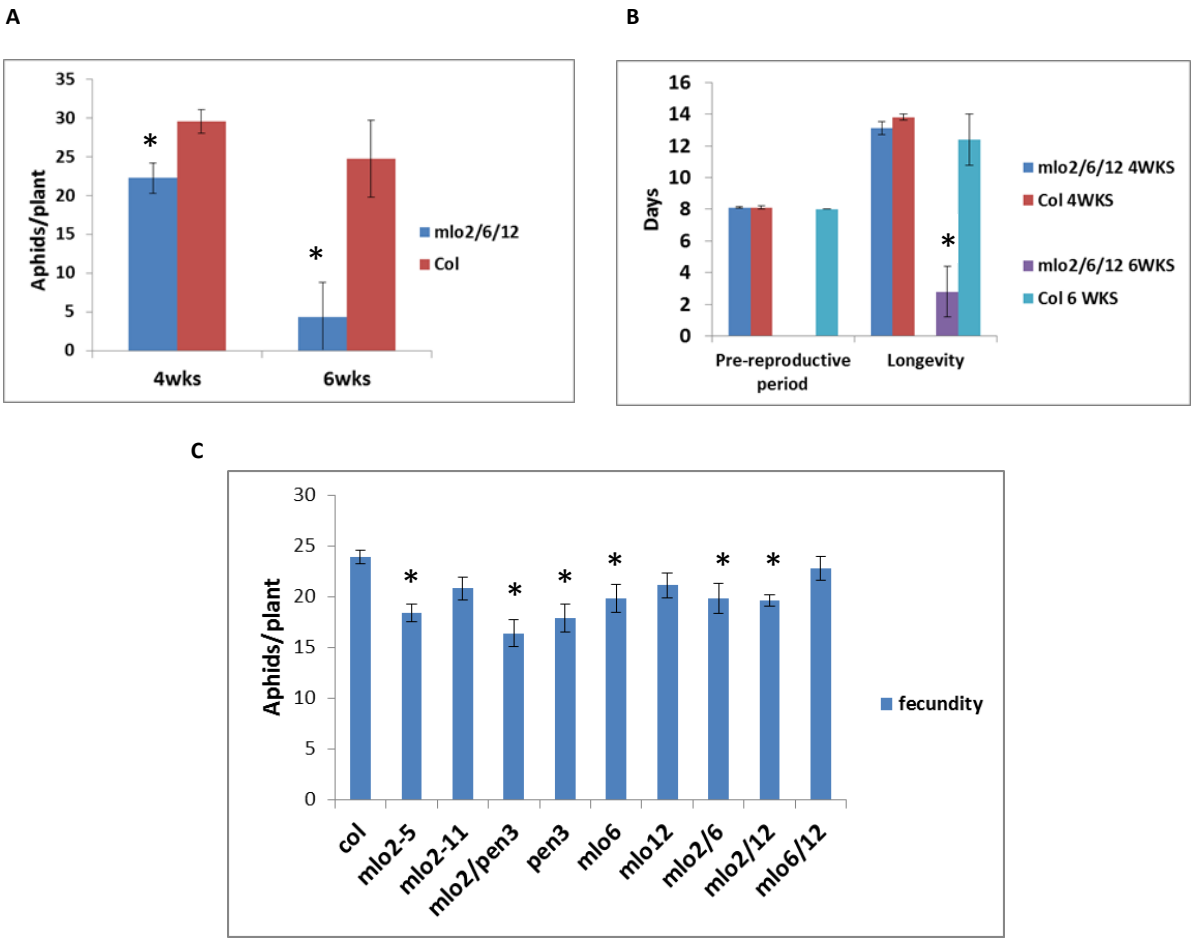


Figure 5: The performance of green peach aphid on Arabidopsis *mlo* mutants and the *pen 3* mutant (*mlo2/mlo6/mlo12, mlo2/6, mlo2/12, mlo6/12, mlo2/pen3, mlo2-5* (*MLO2* knock out line), *mlo2-11* (*MLO2* knock down line*), mlo6,* and *mlo12* compared to wild-type plants. Besides, *mlo2-11* mutant, all the mutants are knock-outs. *MLO2* gene in the double mutants *mlo2/6,mlo2/12*,and *mlo2/pen3* has been knocked-out.

A. **GPA performance (Fecundity):**The number of nymphs borne by each of the introduced aphid on WT plants and the *mlo2/6/12* mutant in a 14 day period in a non-choice test. Each plant received one nymph on the adaxial side of the leaf. This test was carried out on plants aged 4 wks and 6wks. The number of aphids was obtained by getting the average number of nymphs in the replicates used in each of the experiments. The columns represent SE±mean generated at a confidence level of 95%.

B. **GPA performance(pre-reproductive period, longevity):**The average number of days it took for the newly introduced nymphs to reach reproductive maturity (pre-reproductive period) on mutants and WT plants and the survival of the nymphs within 14 days (longevity) on mutants and WT plants. This was tested on 4wks old plants and 6wks old plants. The columns represent SE±mean generated at a confidence level of 95%.

C. **GPA performance (Fecundity):** The mean number of nymphs borne by each aphid introduced on *mlo* single and double mutants, *pen3* mutant, *mlo2/pen3* double mutant and wild-type plants aged 4weeks in a non-choice test . One day old nymph was introduced on the adaxial side of one leaf in the plants used in the experiment. The counts were taken 14 days after nymphs' introduction.

Asterisks (*) show significance difference from WT ($p \leq 0.05$).The columns represent SE±mean generated at a confidence level of 95%.

iii). Host plant selection and host suitability of Activation tag mutants

Dual choice test showed that the aphids had no preference for either lines 1540, 1547, 2119, 2160 or WT plants (Figure 6A). There was no significant difference in the number of aphids recorded on lines 1540 (χ^2=0.01,d.f=1, p=0.927), 1547 (χ^2=0.22,d.f=1, p=0.639), 2119 (χ^2=2.20,d.f=1, p=0.138), and 2160 (χ^2=0.42,d.f=1, p=0.516)relative to WT plants. On the contrary, the preference of aphids for WT plants relative to line 3790 aphids was significant (χ^2=8.82, d.f=1, p=0.003). In non-choice test, longevity of the aphids on activation tag mutants within 14 days ranged from 13.4 to 14 days, while pre-reproductive period ranged from 7.9 to 8.2 days. This was not significantly different from WT plants (ANOVA, p≤0.05). Fecundity of aphids was significantly lower on activation tag insertion line 3790 when compared to WT plants (ANOVA,$F_{1,33}$=15.24,P=<.001;Figure 6B). It recorded a 34% lower number of newly borne nymphs relative to WT plants. Activation tag insertion lines, 2119,2160,1547,1540 on the other hand recorded (3%, 0.7%, 3.8%, 23.5% respectively) lower number of newly borne nymphs, which was not significantly different from the WT 2119-(ANOVA,$F_{1,39}$=0.24p=0.624), 2160-(ANOVA,$F_{1,39}$=0.01,p=0.904), 1547-(ANOVA,$F_{1,39}$=0.30,p=0.585), and 1540- (ANOVA, $F_{1,33}$=3.1,p=0.088).

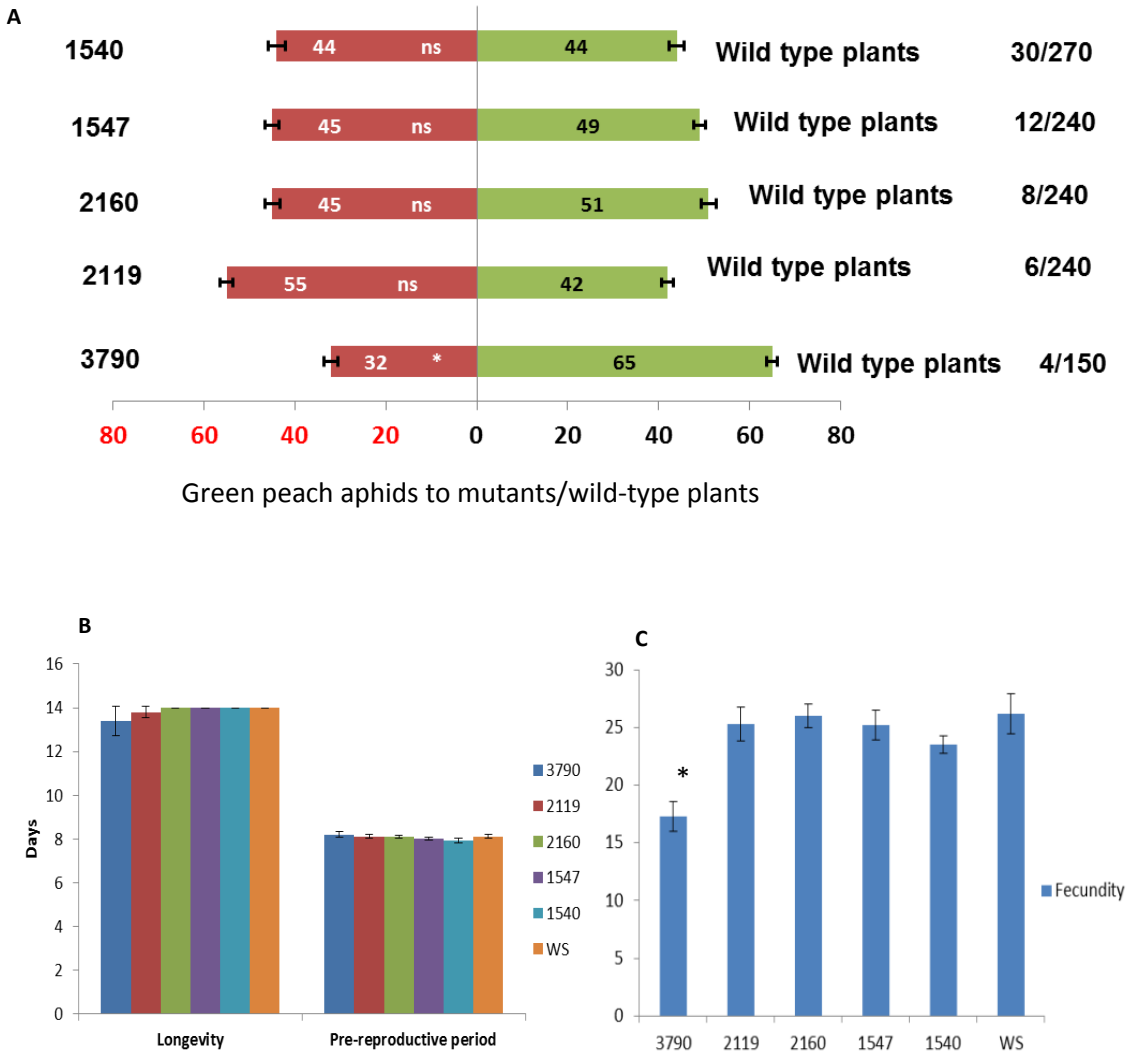


Figure 6: Host plant selection and performance of green peach aphids on Activation tag insertion lines 3790, 2119,1547,1540,2160

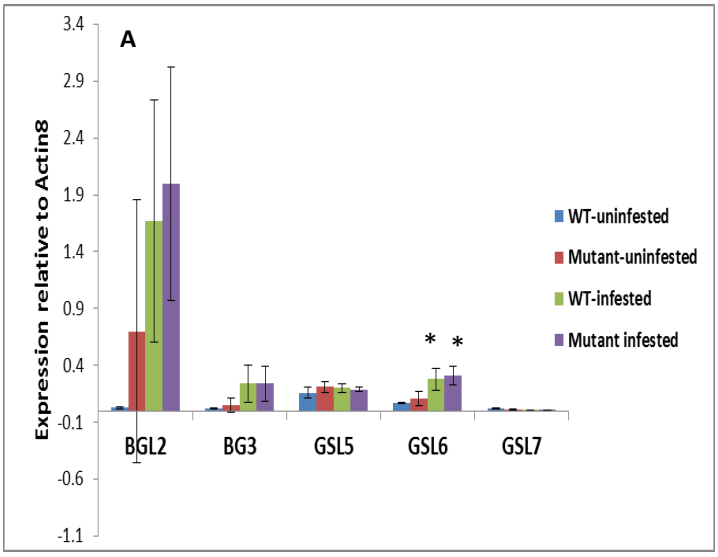
A. **Host plant selection :**The number of aphids expressed in percentage (%) that made a choice for the mutants (red bars) and corresponding WT (green bars) in dual-choice test conducted in arena. Thirty apterous adult aphids were introduced in the middle of arena containing six 4 wks old plants(3 wild-type and 3 plants from one mutant). They were then allowed to make a choice within 24hrs. Total numbers of aphids on the mutants and wild-type plants were then counted. Asterix(*) shows significance difference from WT while (ns) show no significance difference from WT at p≤0.05. The numbers of aphids that did not make a choice and the total numbers of aphids tested are shown at the far right of the figure. The bars represent SE±mean generated at a confidence level of 95%.

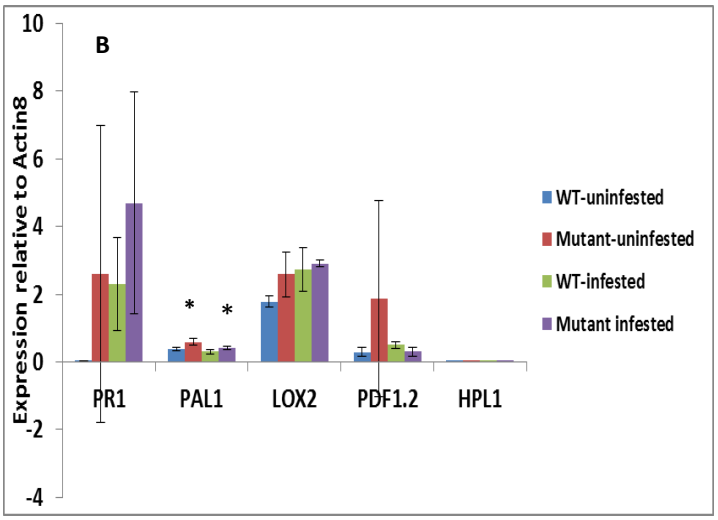
B. **GPA performance (Pre-reproductive period, longevity):**The average number of days it took for the newly introduced one day old nymphs to reach reproductive maturity (pre-reproductive period) on activation tag lines and WT plants and the survival of the nymphs within 14 days (longevity) on activation tag lines and WT plants. The columns represent SE±mean generated at a confidence level of 95%.

C. **GPA performance (Fecundity):**The number of nymphs borne by each of the one day old introduced nymph on WT plants and activation tag lines in a 14 day period in a non-choice test. The number of aphids was obtained by getting the average number of nymphs/treatment in the experiment. The columns represent SE±mean generated at a confidence level of 95%. Asterix(*) shows significance difference from WT (p≤0.05).

iv).Gene expression analysis

Based on the results from the non-choice test, we went ahead to characterise the resistance mechanism in the *mlo2-5* mutant. We studied the expression of the callose metabolism genes, *SAGs* and marker genes for the SA and JA pathway. The expression of callose metabolism genes was not different between the mutant and wild-type plants (Figure 7A). There was no significance difference in the transcripts abundance of *BGL2* (ANOVA, $F_{3,9}$=4.11,p=0.067),*BG3,*(ANOVA, $F_{3,9}$=4.21,p=0.064), *GSL5* (ANOVA, $F_{3,8}$=0.55,p=0.671) *GSL7* (ANOVA, $F_{3,9}$=3.77,p=0.078) in all the treatments. *GSL6* showed a significantly higher expression in infested plants compared to uninfested plants (ANOVA, $F_{3,8}$=7.35, p=0.028); infested mutant had the gene expressed 2.8 fold higher relative to uninfested mutant while the infested wild-type plants had *GSL6* expressed 4 fold higher than uninfested wild-type plants. There was no significant difference in the induction of the JA pathway in the treatments tested (Figure 7B). This is shown by expression of genes *LOX2* (ANOVA, $F_{3,9}$=3.39,p=0.095), *PDF1.2* (ANOVA, $F_{3,9}$=0.28,p=0.84) *HPL1*(ANOVA, $F_{3,9}$=0.67,p=0.603) which was not significant in all the treatments. SA pathway on the other hand was differentially regulated between the mutant and wild-type plants (Figure 7C). The PR1 expression was not significantly different between the treatments (ANOVA, $F_{3,9}$=2.39,p=0.168), while *PAL1* expression was significantly higher in the mutant (both infested and uninfested) compared to wild-type plants (ANOVA, $F_{3,9}$=6.09,p=0.03).The treatments in this experiment had different expression of the SAGs. (Figure 7c). The *SAG13* expression was not significantly different expressed across the treatments (ANOVA, $F_{3,9}$=3.79,p=0.078). On the other hand, *SAG21* expression was significantly lower in uninfested wild-type plants relative to the other treatments (ANOVA, $F_{3,9}$=14.98,p=0.003).





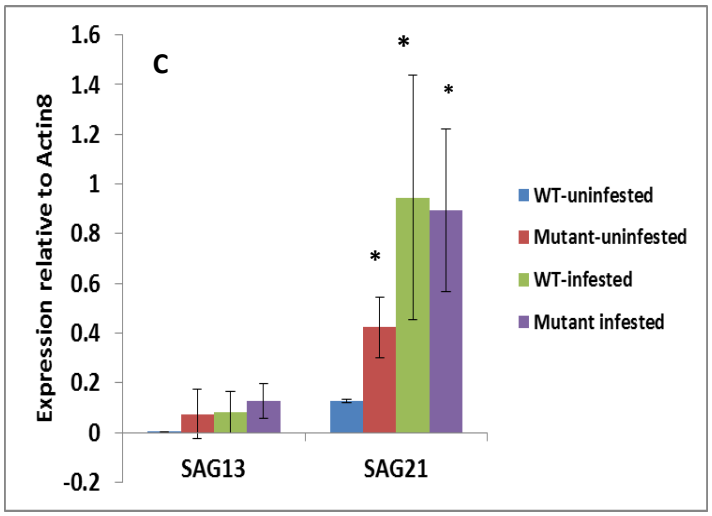


Figure 7: Induced transcripts levels of callose metabolism genes, defense genes in JA and SA pathways, and SAGs (Senescence associated genes) in Arabidopsis *mlo-2* mutant and WT plants in the Columbia (Col) background after 48h of green peach aphid (GPA) feeding quantified with quantitative- real time-polymerase chain reaction (q-RT-PCR). The infested plants had 50 apterous aphids of assorted ages while the uninfested plants were aphid free. This experiment had 3 replicates with 3 plants pooled in each replicate. The quantification of transcript levels of the genes is presented relative to *Actin8*.

A. Expression of callose metabolism genes comprised of callose biosynthesis genes (*GSL5, GSL6, GSL7*) and callose degradation genes (*BGL2, BG3*). The columns represent mean±SD; N=3). Asterix (*) show significant difference in the transcript levels of the genes (one-way ANOVA, LSD, $P<0.05$)

B. Expression of JA (LOX2*, HPL1, PDF1.2*) and SA (*PR1, PAL1*) pathways marker genes. The columns represent mean±SD; N=3). Asterix (*) show significant difference in the transcript levels of the genes (one-way ANOVA, LSD, $P<0.05$)

C. The expression of senescence associated genes (SAGs). The columns represent mean±SD; N=3). Asterix (*) show significant difference in the transcript levels of the genes (one-way ANOVA, LSD, $P<0.05$).

Discussion

i).*Glutathione transferase 1 (GST1)* does not affect GPA's host plant selection and host plant suitability

Our experiment results demonstrate that *GST1* does not affect GPA's host preference and host suitability. *GST1* belongs to the glutathione transferases family (*GSTs*) also referred to as peroxidases. This gene family is induced in response to both biotic and abiotic factors(Reymond et al., 2000; Wagner et al., 2002). They play a role in reactive oxygen species (ROS) detoxification. Transcriptional analysis of plants' responses following aphid feeding suggest that ROS production controls defense responses (Broekgaarden et al., 2008; Kerchev et al., 2012). Boyko et al., (2006) suggested that ROS acquired by aphids during probing, may affect their midgut tissues. ROS also act as defense signals (Bi and Felton, 1995) when a plant is under attack. We therefore expected the *gst1* mutant to be more resistant compared to wild-type plants because of accumulation of ROS in the mutant, but this was not the case. Even though the mutant has reduced detoxification mechanism, the aphids probably compensate for this by producing *GSTs* which they inject into the plant via their stylet (Ramsey et al., 2007*)*. This strategy enables aphids to withstand high ROS accumulation in plants' tissues. Another plausible explanation would be the functional redundancy that exists in members of *GSTs'* in Arabidopsis (Sappl et al., 2009), meaning that another *GST* would take over the function of *GST1* when it is knocked out. It is documented that *GSTs* induction is seen in resistant sorghum when infested with *Schizaphis graminum* hence implicated to be involved in defense response(Zhu-Salzman et al., 2004; Park et al., 2006). Members of the *GST1* help maintain the integrity of plant cells preventing necrosis that could occur as a result of cell death which is caused by increased ROS production. Although an efficient ROS detoxification system is necessary for plant survival, a plant has to maintain a balance between ROS production and its detoxification. High levels of ROS could lead to cell death and inefficient ROS accumulation could result in increased susceptibility (Ni et al., 2001). Hence by the gene bringing about stability by preventing plant tissue damage (Thompson and Goggin, 2006), it could play a role in plant tolerance (Gutsche et al., 2009). Moreover, since *GST1* is implicated to be involved in plant tolerance, plant biomass would have been an additional parameter to monitor in this experiment. These results were obtained from one experiment and we recommend two more experiments with the same set -up to be entirely sure of the tolerance *GST1* confers to plants under aphid infestation.

ii).*Early responsive dehydration 5 (ERD5)* does not affect GPA's host plant selection and host plant suitability

Our results suggest that *ERD5* does not contribute to antibiosis or antixenosis resistance against GPA. *ERD5* also referred to as *proline dehydrogenase 1* is up-regulated in Arabidopsis in response to water stress. Plants accumulate proline during water stress and the osmotic stress associated with increased proline results in the induction of genes to regulate proline in plant tissues. Aphid feeding is thought to decrease the water potential of a plant(Cabrera et al., 1994; Nabity et al., 2009). According to Yahraus et al., (1995), the defense signaling in a plant can be induced as a result of very small changes in turgor pressure in the plant. Reduced turgor pressure leads to induction of drought, salt, low temperature responsive genes (DRT), inclusive of *ERD5*.This gene is seen to be up-regulated in Arabidopsis plants after aphids feeding (Dixon et al., 2005). Since we did not see the behaviour and performance of aphids being affected by this gene, we hypothesise that the induction of this gene during aphid feeding could be purely due to water stress that is perceived by the plant when the aphid damages plant tissues during feeding. Thus, ERD5, could be implicated to play a role in plant tolerance.

iii).Senescence associated gene 21 (SAG21) contributes to antixenosis against GPA in Arabidopsis

Antixenosis resistance against GPA in Arabidopsis conferred by *SAG21* was demonstrated in this study. Aphids preferred settling on *sag21* mutants over WT plants. The involvement of volatiles in this resistance mechanism is not known since Y-tube olfactometer experiment was not successful. It has been demonstrated that GPA feeding on Arabidopsis plants induces premature senescence which limits the reproductive potential of aphids (Pegadaraju et al., 2005). Hence, the preference of aphids for *sag21* mutant in our experiment could be as a result of reduced nutrients flow to the infested leaves of WT plants. Premature senescence incapacitate aphids from manipulating sink-source relationship (Pegadaraju *et al.*, 2005). Hence aphids are deprived of essential nutrients for their survival. The transcriptome of a senescencing leaf consists of a large number of defense related genes (Andersson *et al.*, 2004) and thus the preference of aphids for the non-senescent *sag21* mutant plants is justifiable. A study conducted on barley, demonstrated more ethylene evolution in resistant plants compared to susceptible plants upon aphid infestation (Argandona et al., 2001). Ethylene is known to promote senescence. It could be that the resistance in barley plants to the aphids was linked to senescence. The performance of GPA in the non-choice test was not affected in *sag21* mutant. This could be because *SAG21* is just one of the molecular marker genes for the onset of senescence in plants and that other

SAGs (*SAG13 and SAG27)* also play a role in premature leaf senescence. These other *SAG* genes could have additive effects to the functioning of *SAG21* in the induction of premature leaf senescence. The limited physiological changes in the plant caused by knocking-out *SAG21* could not have been sufficient to positively influence aphids' performance on *sag21* mutants in non-choice test. Other studies suggest that aphids induce premature leaf senescence in susceptible plants to increase the translocation and breakdown of leaf proteins (Burd, 2002). This phenomenon is quite favourable to aphids since the nutrients are easily available. Moreover, Alvarez et al., (2006) found that the performance of green peach aphids on *S.tuberosum* cv. kardal is dependent on foliage maturity of the plant. The old senescent leaves were more susceptible to aphids. This disparity could have been brought about by different plants used in the two experiments. We worked with Arabidopsis plants, while Alvarez et al., (2006) worked with *Solanum* sp*.* We therefore propose that *SAG21* only has an antixenosis effect to GPA in Arabidopsis. Since these are results from one trial, we recommend a repetition of this experiment to confirm the results.

iv).Activation tag mutants of Arabidopsis differentially affect GPA's preference and performance

From dual choice tests and non-choice tests, activation tag mutant line 3790 showed both antibiotic and antixenotic effects on the behaviour of green peach aphids. The resistance mechanism of 3790 activation tag mutant needs to be characterised. Using EPG in characterising this mutant would help identify the exact location where the resistance is based. At this point we cannot conclude if the antixenosis effect of 3790 activation tag mutant to green peach aphid is due to volatiles or toxic secondary metabolites. Y-tube experiment needs to be conducted to explain this phenomenon.

v).*Mildew locus O (MLO)* genes differentially modulate resistance against GPA in *Arabidopsis thaliana*

The *mlo2/mlo6/mlo12* triple mutant is demonstrated in our study to have antibiotic potential against GPA. This resistance seems to be age dependent since the triple mutant was more resistant at the age of six weeks compared to four weeks. Our results show that *MLO2* and *MLO6* negatively affect this resistance. However, we did not see *mlo2-11* RNAi line negatively influencing GPA's fecundity. *MLO2* protein could still have been abundant in this line. Our results demonstrate *MLO2* as being the major gene influencing the susceptibility of Arabidopsis to GPA. This is shown by the *mlo2/mlo12* mutant which is resistant to GPA and the resistance comparable to mlo2-5 resistance. The *MLO12* does not

increase the susceptibility of Arabidopsis to GPA, and there is a probability that it could interact with *MLO6*. This is demonstrated in our results; WT susceptible phenotype was reverted in the *mlo6/mlo12* double mutants. *Mlo2, Mlo6* and *Mlo12* genes in *Arabidopsis thaliana* have been reported to have unequal genetic redundancy in regulating defense response against the powdery mildew pathogen (Consonni et al., 2010). To our knowledge, no studies have been conducted to ascertain the role these genes play in modulating defense response against insects. Studies previously conducted on *mlo2* mutant show that six-weeks old mutants have spontaneous callose deposition, early senescence, and a high rate of glucosinolates turnover (Consonni *et al.*, 2006). These characteristics of the mutant increase with age since the mutant is characterised by spontaneous callose deposition and increased senescence in the later stages of its development. The age dependent resistance observed in this study could be attributed to any of these characteristics of the mutant. Glucosinolates in *mlo2* and *mlo2/mlo6/mlo12* mutants are broken down by atypical myrosinase *PEN2* and the breakdown products are transported by *PEN3* to the apoplast (Consonni *et al.*, 2006). It has been demonstrated that GPA performance on the *pen2* mutant is the same as on WT plants (De Vos et al., 2005). Therefore, the presence of glucosinolate breakdown products in the *mlo2* mutant does not explain the resistance of the mutant to GPA. GPA feeds on photoassimilates in the phloem and their mode of feeding enable them to evade the toxic glucosinolate products in this mutant which are localised in the apoplast.

Our results show that GPA did not induce considerable amounts of callose metabolism gene in the *mlo-2-5* mutant relative to WT plants. However, we cannot rule out callose from being the cause of this resistance since we only sampled material for qPCR at one time point. We therefore might have missed out on sampling at the right moment when callose metabolism genes were being expressed. Uninfested mutants did not have significant levels of callose metabolism gene transcripts compared to uninfested wild-type plants. This is expected since the mutant is programmed to spontaneously accumulate callose at six weeks. From the qPCR results, *GSL6* is highly upregulated in infested plants compared to uninfested plants, a result which is corroborated by Kusnierczyk et al., (2008). There is therefore the need to study gene expression of callose metabolism genes at several time points after aphids' infestation. Callose staining would also be a confirmatory test to investigate the differential callose deposition on the mutants and WT plants in the case that callose is regulated at the protein level. Positive finding from callose staining experiment will also corroborate our hypothesis of callose being involved in the mutants'resistance to GPA.

The *mlo 2* and *mlo2/ mlo6/ mlo12* mutants present an early senescence phenotype which is evident by necrotic and chlorotic spots that show on the leaves (Figure 8). *Mlo* genes are negative regulators of senescence (Consonni et al., 2010) and knocking out these genes results in the senescence phenotype. From qPCR results, *SAG21* which is a marker gene for senescence, was significantly up-regulated in infested plants compared to uninfested plants, with uninfested wild-type plants having very low SAG21 transcripts. The up-regulation of *SAG21* already present in the uninfested mutant is an indication that senescence plays a role in resistance conferred by *mlo-2-5* mutant. Moreover, SA is significantly up-regulated in infested mutant relative to infested WT plants. This is evidenced by more *PAL1* transcripts present in *mlo2* mutants relative to WT plants from the qPCR results. The senescent phenotype of this mutant has been shown to be dependent on SA accumulation and not on JA or ET(Consonni et al., 2006). Previous studies also show that *mlo2* mutant has increased *SAG13* transcript abundance (Consonni et al., 2010). In previous microarray studies, *SAG13* was seen to be up-regulated in Arabidopsis after feeding by GPA (Kerchev et al., 2012; Pegadaraju et al., 2005) as a defense mechanism. According to Pegadaraju et al., (2005), aphids induce early senescence in plants which results in resource mobilization away from the infested leaves. This in turn reduces nutrient availability for the aphid, translating to lower reproductive potential.

The *pen3* mutant was also seen to be resistant to GPA in this study. This mutant has spontaneous callose deposition and early cell death (Stein et al., 2006), characteristics it shares with *mlo2 and mlo2/mlo6/mlo12* mutants. Therefore, we hypothesize that aphid resistance in *mlo2-5* and *mlo2/mlo6/mlo12* mutants could be associated with the early senescence phenotype and spontaneous callose deposition they exhibit. These results are not conclusive and more studies need to be done on the mutants to elaborately dissect the resistance mechanism they present towards GPA.

Conclusion and future research

This project has identified the *MLO2* and the *MLO6* as being the susceptibility genes in Arabidopsis that confer susceptibility to GPA. The resistance in the *mlo* mutants is linked to SA acid accumulation in plant tissues, callose deposition and premature leaf senescence. It would therefore be interesting to cross these *mlo* mutants with mutants deficient in the SA pathway and monitor GPA performance on the resulting mutants. Transcriptional studies of senescent Arabidopsis leaves would be useful in identifying components in the senescent leaves that negatively affect the performance GPA. This can then give information on callose deposition and premature leaf senescence role in resistance to GPA in this mutant. The *mlo-2-5* mutant showed a high *PAL1* transcript accumulation. We recommend a study be done to study the effect of phenolics on the performance of *M. persicae*. For aphid infestation on plant material to be used for qPCR, the infestation levels should be increased, or the use of clip-on cages should be employed to confine aphids within individual leaves. This might increase the transcriptional responses observed in our results.

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Appendices

Table 2: Primers sequence

Gene name	Primers	Source
Actin 8 (At1g49240)	F5'- CCCAAAAGCCAACAGAGAGA-3' R5'-CATCACCAGAGTCCAACACAAT- 3'	(Lilly, 2011)
GSL5 (At4g03550)	F5'-CTGGAATGCTGTTGTCTCTGTTG-3' R5'-TCGCCTTTTGATTTCTTCCCAGT-3'	(Jacobs et al., 2003)
GSL6 (At1G05570)	F 5'-GAAGGGTTTGGGCGTTGGAAG-3' R 5'-CAATGAGAAGCATTCCCCATCCAGTT-3'	(Jacobs et al., 2003)
GSL7 (At1g06490)	F 5'-GAATGTTGCGGTCCAGATTT-3' R 5'-TCTGTGAGAACCTCGCAATG-3'	Primer 3 plus
BGL2 (At3g57260)	F 5'- TGGCAAGGTATCGCCTAGCATC -3' R 5'- AGCTTCCTTCTTCAACCACACAGC -3'	(Brotman et al., 2012)
BG3 (At3g57240)	F 5'- GGGTCGCAGGTTGTTTCCATTTC-3' R 5'- TCCTTCTTCGACACGGCAGTTG-3'	(Brotman et al., 2012)
SAG13 (At2g29350)	F 5'-CTCGTGACCAACGAGTGAAA-3' R 5'-CCACATTGTTGACGAGGATG-3'	(De Vos and Jander, 2009a)
Sag 21 (At4g02380)	F 5'-TGGGTAATGATCTGGTGCAG-3' F 5'-GAGGAAACCAAACGCTTCTC-3'	(Brosche and Kangasjarvi, 2012)
PR1(At2g14610)	F 5'-CTGCAGACTCATACA-3' F 5'-ATCCGAGTCTCACTGACTTTC-3'	(Snoeren et al., 2011)
PAL1(At2g37040)	F 5'-TGTAGCGCAACGTACC -3' R 5'-GTTCGGGATAGCCGATG -3'	(Snoeren et al., 2011)
LOX2(At3g45140)	F 5'-ACAACTAAGTGCCATGGATCC -3' R 5'-GTAAGCCTTCCTGGTCAAACC -3'	(Snoeren et al., 2011)
HPL1(At4g15440)	F 5'-GGCGTTCGTGTTGGAGTTTATC -3' R 5'-GGATTCGATTGTTCCCCAGAA -3'	(Snoeren et al., 2011)
PDF1.2(At5g44420)	F5'CACCCTTATCTTCGCTGCTC3' R5' GCACAACTTCTGTGCTTCCA3'	Primer 3 plus

Figure 8: mlo mutant phenotypes. Four weeks old *Arabidopsis thaliana* wild type plants, mlo2/pen3-1 (Upper row from left) pen3-1, and six weeks mlo2, mlo6, mlo 12 mutants (Bottom row from left). Yellowing of leaves is more apparent on mlo2/pen3 mutants at four weeks and mlo2, mlo6, mlo12 triple mutant at six weeks.

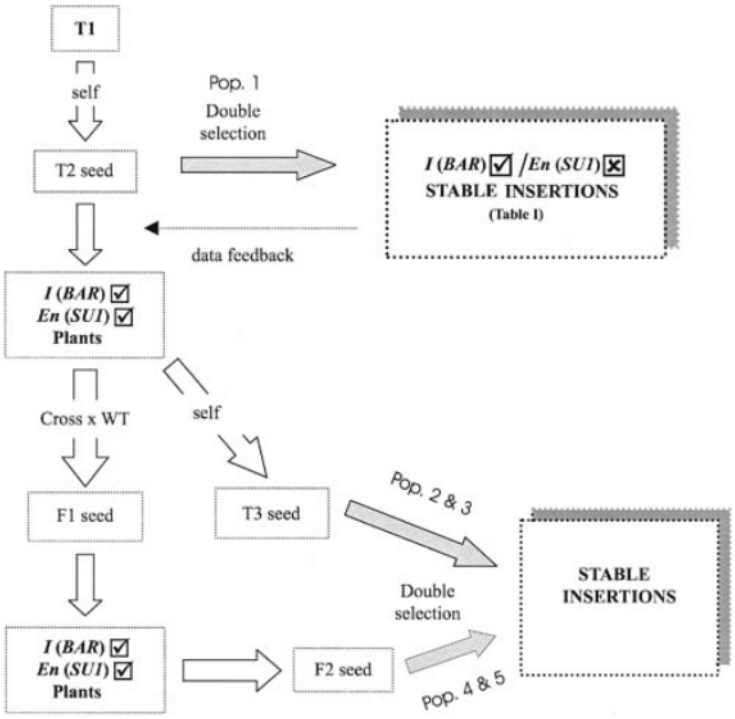

Figure 9: Generating a population of stable activation tag insertions.
T1 contains the transformation construct with activation elements and selectable markers. Selection of stable insertion lines is carried out from subsequent selfing populations or using F2 seeds as parental lines. Source:(Marsch-Martinez et al., 2002)