



WAGENINGEN UNIVERSITY & RESEARCH  
PLANT BREEDING: INSECT RESISTANCE GROUP  
MSc PLANT SCIENCES THESIS PROJECT

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Characterisation of Aphid Resistance in an *Arabidopsis*  
Activation-Tag Mutant Line

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## 1.0 General Introduction

### 1.1 The Herbivore: Green Peach aphid

Phloem feeding insects (PFI) constitute one of the most important feeding guilds of herbivorous insects, with aphids making up the largest group. The hemipteran Green peach aphid (GPA); *Myzus persicae* (Sulzer), is a polyphagous herbivore with an exceptionally wide host range of over 50 plant families (Blackman et al., 2007). Unlike the generalist GPA, the specialist cabbage aphid; *Brevicoryne brassicae* (Linnaeus), feeds only on plants of the *Cruciferae* family, however, mainly in the *Brassica* genus. Aphids are known to cause devastating damage to Agricultural crops through the transmission of virus diseases as they serve as vectors of viruses of economic importance (Kennedy et al., 1962; Pares, 1992). Using their stylets, they are able to suck large quantities of photoassimilates from phloem sieve elements thus depriving the plant of nutrition, eventually leading to chlorosis and stunted growth (Miles, 1999; Pollard, 1973). The GPA has been reported to alter host source-sink patterns in *Arabidopsis thaliana* (Singh et al., 2011). Aphid infested leaves show an increase in the monosaccharide H<sup>+</sup> symporter mRNA levels (Moran and Thompson, 2001) suggesting that the herbivore manipulates its host's physiology to redirect nutrients to the feeding site. Using their stylets, GPAs are able to cause direct physical damage to plant tissue during probing. The carbohydrate-rich phloem sap causes the aphid to excrete excess sugars as honeydew. This honeydew serves as a nutritional substrate for fungi which in turn produce a black sooty mould. The fungal mould developing on the plant surface prevents photosynthetic tissue from receiving light and also gives crop produce an unsightly appearance (Sandström and Moran, 1999).

Pesticides such as dimethylcarbamates are currently being used to control aphids, however, it is well documented that aphids can easily mutate into pesticide-resistant strains (Roush and McKenzie, 1987). Furthermore, pesticides are environmentally unfriendly and are known to destroy beneficial insects like pollinators and natural enemies of pest insects. Biological control is also employed for aphid control with the use of natural enemies like the lady bugs and lacewings (Oka, 1991) but due to the exponential growth rates of aphid populations, it is unlikely that they can be fully controlled (Vásquez et al., 2006). The shortfalls of the above-mentioned control measures have left the best option for aphid control to be the development and cultivation of aphid resistant cultivars. Several plant resistance genes (R) have been shown to confer GPA resistance e.g. Mi 1.2 from *lycopersicon peruvianum* (wild tomato) and Vat gene from melon conferring resistance to *Macrosiphum euphorbiae* (potato aphid) (Howe and Jander, 2008; Walling, 2008) and *Aphis gossypii* (cotton aphid) (Villada et al., 2009) respectively.

## 1.2 Host Plant Defence

Plant defence is firstly classified as either being direct or indirect. Direct defence involves the host plant interacting directly with the herbivore and includes the use of physical barriers such as leaf toughness and glandular trichomes. Direct defence may also be through chemical barriers such as phytotoxins e.g. glucosinolates (GS), antidigestive proteins (e.g. protease inhibitors) and other traits that are deterrent or toxic to herbivores (Howe and Jander, 2008). Indirect defence involves the host plant employing the assistance of other organisms of higher trophic levels which are natural enemies to the herbivore. This form of protection is afforded by herbivore induced plant volatiles (HIPV) and nectar rewards from extra-floral nectaries (EFN) which attract the predators and parasitoids (Kessler and Baldwin, 2001). Direct or indirect defences could either be constitutive or induced; depending on whether the defence mechanisms are operative before (constitutive) or after (induced) herbivore attack.

Host plant defences can be further classified as being antibiotic and antixenotic direct defence mechanisms against herbivory in which the insect physiology (i.e. fecundity and growth) is restricted in the former and feeding as well as settling is deterred in the latter. These mechanisms can be triggered as the aphid merely crawls over the plant before probing the plant surface (Bown et al., 2002) or as its stylet finds its way to the phloem before finally establishing its feeding site at the sieve element phase (Pegadaraju et al., 2007). Sealing of sieve elements by coagulated phloem protein and/or callose depositions is in response to stylet penetration in phloem vessels and hinders sap uptake (Will and van Bel, 2008). The PAD4 protein in *A.thaliana* accession Wassilewskija (Ws) and Landberg erecta (Ler) has been implicated in modulation of phloem-based antixenotic and antibiotic defence against GPA by limiting sap uptake thus serving as a deterrent and restricting the aphid population (Pegadaraju et al., 2007). A number of studies in aphid-plant systems have shown reduced phloem sap ingestion to be associated with host plant resistance (Tjallingii, 2006) ; (Kaloshian et al., 2000; Klingler et al., 2005).

The production of volatile and non-volatile secondary metabolites is reported to be altered in response to recognition of aphid attack and these include GS, phenolics and terpenoids (Mewis et al., 2006). Glucosinolates have been found exclusively in the *Brassicaceae* family and products of their hydrolysis, such as thiocyanates, play a role in defence and act as attractants of natural enemies of herbivorous insects (Halkier and Gershenzon, 2006). Some are released by glandular trichomes on the leaf surface before stylet penetration whilst toxic metabolites like thiocyanates are released after tissue damage (Pegadaraju et al., 2007; Rask et al., 2000).

### 1.3 Signalling Pathways Involved in the Arabidopsis-Aphid System

For induced defence to be effective, the host plant must be able to recognise and respond defensively to the herbivore thus conferring immunity. Very little is known about molecular recognition during aphid- host plant interactions but it is assumed that peroxidases and pectinases found in aphid saliva could be elicitors of defence or the products of aphid endosymbiotic bacteria *Buchnera aphidicola*, which lives inside aphids in specialized cells called bacteriocytes (Smith and Boyko, 2007). In a much recent study, three candidate effectors from aphid saliva have been identified which showed a suppression of plant defense responses and/or aphid reproductive performance which include Mp10, Mp42 and MpC002. Both Mp10 and Mp42 induced chlorosis and local cell death thus reducing aphid performance whereas MpC002 enhanced aphid fecundity (Bos et al., 2010).

Induced defence during aphid-Arabidopsis interactions is facilitated by signal transduction pathways involving three major phytohormones namely: jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Zhu-Salzman et al., 2004). Microarray studies on the transcriptional responses of *M. persicae*-Arabidopsis interactions, as is the case with most phloem feeders, have revealed a significantly higher expression of the SA signal pathway (  $\beta$ -1-3-glucanase and PR1) as compared to those of the JA/ethylene (antimicrobial defensin PDF1.2) and of the major insect response/wound pathways ( PI and LOX2 ). It has been postulated that the limited elicitation of JA-mediated defence responses could be due to minimised tissue damage by the stealthy feeding behaviour of the aphid or the antagonistic crosstalk with SA (Walling, 2008; Zhu-Salzman et al., 2004).

### 1.4 Activation Tagging

Activation tagging is a type of insertional mutagenesis in which a T-DNA construct; consisting of a transposable element with strong transcriptional enhancers from the Cauliflower mosaic virus (CaMV) 35S gene, is inserted into an organism's genome and is able to overexpresses genes adjacent to its insertion site (Walden, 2002). Where the creation of knockout mutants using T-DNA and transposons may not be effective in determining gene function, i.e. where the target genes are redundant or may give a lethal phenotype after being knocked out; activation tagging has become useful (Walden, 2002).

A system to generate stable activation tag inserts has been developed in Arabidopsis using a two-component maize *Enhancer-Inhibitor (En-I)* transposon system (Marsch-Martinez et al., 2002). The inserts carrying strong activating sequences act on genes adjacent to the insertion site and overexpress the tagged genes to reveal dominant gain-of function phenotype genes (Speulman et al., 1999; van der Graaff et al., 2000). The observed phenotypes can give clues as to the gene function or the metabolic pathway the gene could be involved (Marsch-Martinez et al., 2002) . It has been reported that genes within a range of 3Kb from the enhancer can be activated to give rise to novel phenotypes (Weigel et al., 2000).

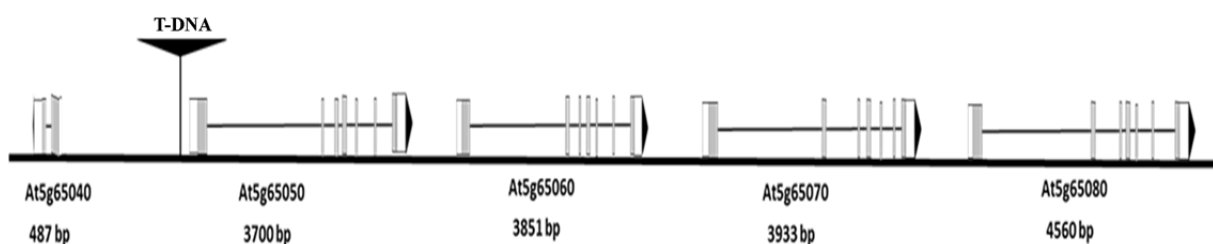
## 1.5 Background Knowledge on The Activation-tagged Arabidopsis Mutant 3646

In a previous study, an Arabidopsis activation tag mutant library (Marsch-Martinez et al., 2002) was screened to identify aphid resistant lines using a high throughput *M. persicae*-Turnip Yellow Virus screening system (Chen et al., 2012). This study was carried out in order to understand the aphid resistance mechanisms in Arabidopsis. A *Ws* ecotype mutant, **3646**, with randomly placed *Zea Mays* En-I transposon insert was one of the mutants that showed aphid resistance as lower population development was observed after 14d.p.i. A slower growth rate was also observed in this mutant line as shown in Fig1. Using inverse PCR, the location of the insertion was found to be in front of the gene At5g65050 as shown in Fig 1 below (Chen et al., unpublished).



**Figure 1:** Aphid resistant mutant line showing slower growth rate than WT Ws (13 weeks after sowing)

The genes flanking the T-DNA insert are arranged in a tight cluster at the bottom of chromosome V (Alvarez-Buylla et al., 2000; Ratcliffe et al., 2003). Genes At5g65050 (MAF2/AGL31), At5g65060 (MAF3/AGL70) and At5g65070 (MAF4/AGL69) are all floral repressors; negatively regulating flowering and vernalisation in Arabidopsis (Fig 2).



**Figure 2: Schematic representation of Arabidopsis (Ws) mutant line 3646 with randomly placed *Zea mays* En-I transposon inserted in front of gene At5g65050.**

These four genes are highly related to the Flowering Locus C (FLC) gene which is a key floral repressor in the vernalisation maintenance (Ratcliffe et al., 2003). The At5g65080 (MAF5/AGL68) gene, on the other hand, is up-regulated during vernalisation and regulates flowering time. However, At5g65040 codes for a protein of unknown biological and molecular function ([www.arabidopsis.org](http://www.arabidopsis.org)).

## 2.0 Objective

Characterization of aphid resistance mechanisms in the Arabidopsis mutant line 3646.

### 2.1 Study questions

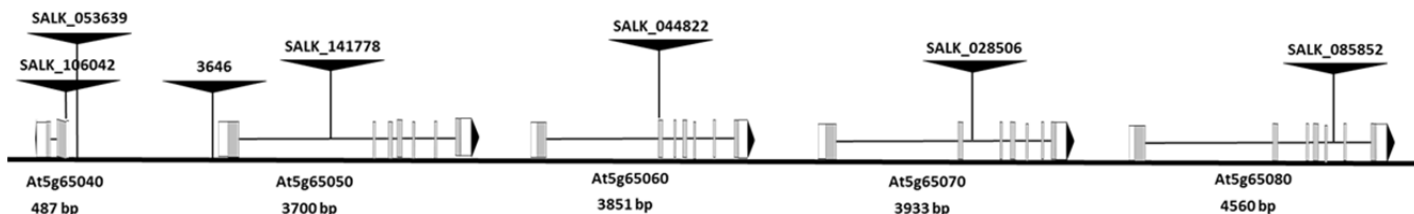
1. Which gene(s) are responsible for the aphid resistance in this mutant line?
2. Which defence signal transduction pathways are up/down regulated?
3. What is the mechanism of aphid resistance in this mutant line?
4. Is there evidence of trade-off between aphid resistance and plant growth?

## 3.0 Materials and Methods

### 3.1 Study System

**Aphids:** Apterous (wingless) forms of the GPA: *M. persicae* (Sulzer) and cabbage aphid *B. brassicae* (Linnaeus).

**Plants:** 3-4 weeks old plants were used in this system: activation tag mutant line 3646, wild type (WT) *A.thaliana* Wassilewskija (Ws) and Colombia (Col-0). Transgenic lines including SALK lines as shown in Fig 3 and Table1 below.



**Figure 3: Diagrammatic representation of the location of the T-DNA inserts on the candidate genes**



**Table 1: T-DNA insertion lines from the Salk collection**

Affected genes	Numbers from NASC database	Salk numbers
at5g65040	N656501	SALK_053639C
at5g65040	N656840	SALK_106042C
at5g65050	N677189	SALK_141778C
at5g65060	N674795	SALK_044822C
at5g65070	N681076	SALK_028506C
at5g65080	N668580	SALK_085852C

### Plant growth and Insect cultures

The plants were grown in a climate room with a regime of photoperiod of 8L:16D, 20°C and 18°C day and night temperatures respectively and 70% relative humidity. The aphids were reared on *Brassica rapa* (Chinese cabbage) in a controlled-environment (20-22°C, photoperiod of 6L: 18D regime).

## 3.2 Aphid Assays

Before the T-DNA tag mutant lines were used, the following tests were done:

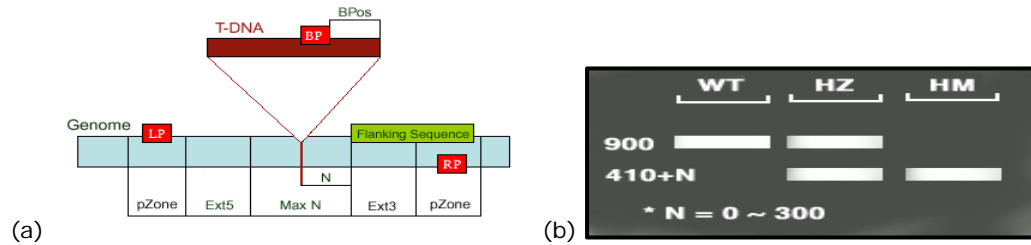
### i. T-DNA knock-out mutant Confirmation

PCR analysis of the candidate genes' expression in leaves of WT Arabidopsis and the SALK lines was done to establish whether the target genes had been completely knocked out. PCR using gene-specific primers (Appendix I and III) and Actin 8 as the reference gene was performed under the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 1 min and 68°C for 2.5 min, with a final extension of 68°C for 7 min.

### ii. Test for Homozygosity in T-DNA knockout mutants

A PCR analysis of the gene product from the leaves of the WT and SALK lines was performed using a 3 primer system as shown in Fig4. Using NaOH gDNA isolation protocol (Wang et al., 1993), young leaf samples were placed into cluster tubes to which 20ul NaOH (0.5M) was added. The samples were shaken twice in the tissue-lyser at a frequency of 25 for 30sec per run. 80ul tris HCL of pH8.0 (100mM) was added to each sample before vortexing and spinning down shortly. Five ul of DNA was transferred to 95 ul Milli-Q water before being used for the PCR. 7ul of each sample was later placed on 1% agarose gel with Tris/Borate/EDTA (TBE) buffer set at 90V for 10 min. PCR using the T-DNA left border primer and one gene specific primer was performed under the following conditions: 95°C

for 5 min, followed by 30 cycles of 95°C for 30 sec, 50°C for 1 min and 72°C for 2.5 min, with a final extension of 72°C for 7 min. The primers used in this system can be referred to Appendix II.



<http://signal.salk.edu/tdnaprimers.2.html>

**Figure 4: T-DNA primer Design**

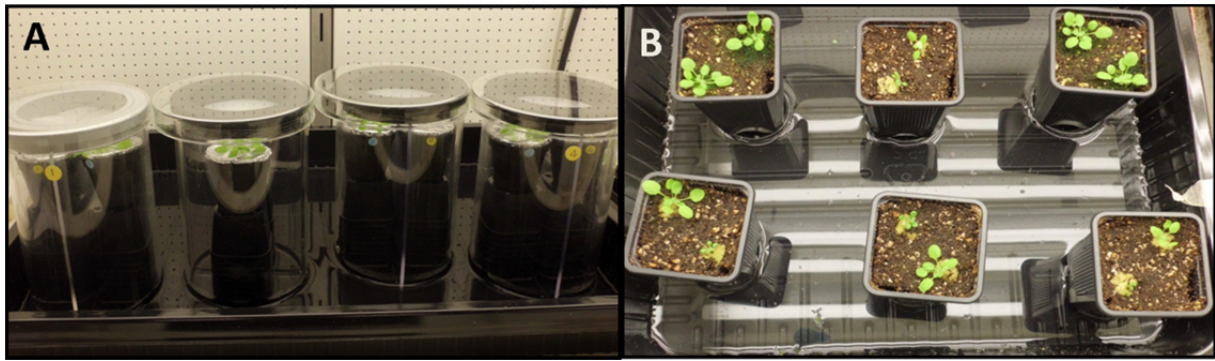
(a) Primer position: LP-left primer, BP-T-DNA border primer, RP-right primer; (b) The expected PCR product output for the WT (wild type), HZ (heterozygous) and HM (homozygous) SALK lines.

### 3.2.1 Dual Choice Test

The antixenotic effect of the WT Arabidopsis and mutant lines was investigated using two dual choice test setups i.e. the cylinder and pot setup. In the cylinder setup, plants were placed on either side of the bi-partitioned cylinder with an air tight lid (see FIG 5-A). The settling preferences of GPA on either 3646 or WT WS Arabidopsis plants was evaluated after 30 adult aphids were released on the fine mesh covering the lid before placing a glass petri dish to prevent them from escaping. Plant volatiles from the genotypes to passing through the mesh are expected to affect aphid choice (Fig 5-A). The total aphid number on either side of the cylinder was recorded after 5 min, 10 min, 15 min, 20 min, 25 min and 30 min. Eight replicates were used for each genotype.

In the pot setup aphids were allowed to come into contact with the plants to make their host choice. One mutant and WT Arabidopsis plant were placed in a single pot diagonally apart. Ten replicates were used for each genotype. Thirty adult aphids were released in the center of the pot and allowed to make a choice between either genotype (FIG5-B). The total aphid number on each plant was recorded after 15min, 30min, 1h, 3h 6h and 24h time points.

**Data Analysis:** Settling preference of the aphids between genotypes was analyzed in the dual choice assay using  $\chi^2$ .



**Figure 5: Dual choice test: Settling behaviour of GPA and cabbage aphid on WT Arabidopsis and mutant plants**

(A) Cylinder setup with 3646 and WT Ws potted plants placed in tightly covered cylinders with a fine mesh on top separating the aphids from them. Aphids are guided to their preferred host choices by the plant volatiles. (B) Pot setup in which aphids are allowed to be in contact with plants as they make their settling choice.

### 3.2.2 Non Choice Test

Antibiotic effects of 3646 mutant line were investigated in the non-choice aphid assay using both the GPA and cabbage aphid, compared against the WT Ws plants. Function verification of each candidate gene likely to be involved in GPA resistance in 3646 mutant was also carried out in this bioassay as aphid performance on WT plants and the T-DNA knockout mutants for each gene (SALK lines) was compared. Adult aphids were synchronized overnight on *Brassica rapa* and 1 day-old nymphs were collected. One of each nymph was placed on the adaxial side of fully expanded Arabidopsis plants. 17 plants per genotype were used and randomly placed in the trays. After every 2 days the trays were turned to ensure that all treatment plants received similar environmental conditions throughout the experiment. The total number of aphids was recorded after 14 days.

**Data Analysis:** Independent-samples *t*-test ( $\alpha=0.05$ ) was used to compare population development between the WT and mutant genotypes in the non-choice aphid assay.

## 3.3 Gene Expression Analysis by qRT-PCR

Quantitative real time-polymerase chain reaction (qRT-PCR) was used to study the transcript levels of the MAF family genes, At5g65040, signalling pathway signature genes of SA, JA and ET as well as ABA and stress responsive genes as shown in Table 2 below.

**Table 2: The target genes for qRT-PCR analysis**

FLANKING GENES	SA MARKERS	JA MARKERS	ET MARKERS	ABA & STRESS RESPONSIVE
At5g65040	PR1	PDF1.2	ERF1	RD22
At5g65050		VSP2		
At5g65060				
At5g65070				
At5g65080				

### 3.3.1 Identification of Candidate Genes

In order to determine which of the flanking genes could be involved in GPA resistance in 3646, gene expression was analysed using qRT-PCR to identify whether there has been an upregulation or not with comparison to the WT plants. 3 weeks old WT (Ws) and the 3646 mutant Arabidopsis plants were used; 3 biological replicates with 17 plants pooled together into one replicate.

RNA extraction was done using RNeasy Plant mini kit (Qiagen). RNA quantification was done using Nano-drop (ISOGEN-Life science) followed by the DNase treatment on 1µg of RNA prior to cDNA synthesis using the iScript cDNA synthesis kit (BIO-RAD). The reaction mixture was placed in a thermocycler using the following program: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, cool down to 4°C. qRT-PCR was performed in a Real-Time thermal Cycler (Optical system software, version 2.0 for MylQ-BIO-RAD) using the following program: 95°C for 3min, 40 cycles of 95°C for 15 sec, and 60°C for 1min. Actin 8 (At1g49240) was used as the reference gene to normalize gene expression across the samples. The gene specific primers for the candidate genes used can be referred to Appendix I.

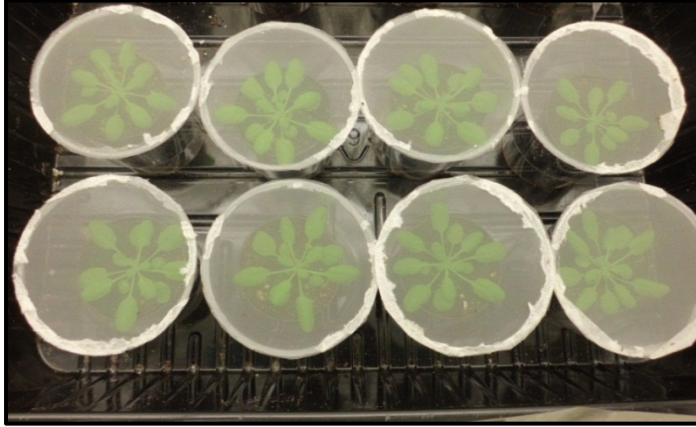
### 3.3.2 Identification of Defence Signalling Pathways Up or Down-Regulated In 3646

To determine which defence signalling pathways were up or down regulated in this particular aphid resistant Arabidopsis mutant, gene expression levels of signature marker genes of the different signal transduction pathways were investigated (Table2). Three-weeks old WT (Ws) and the 3646 mutant Arabidopsis plants were used with three biological replicates for each genotype (Refer to Appendix I for the primers used for q-RT-PCR).

### 3.2.3 Evaluation of Candidate Genes Expression Levels During GPA Infestation

Gene transcript expression levels of the candidate genes were compared between infested and uninfested WT Col-0 Arabidopsis plants to evaluate their expression pattern during GPA infestation. 15 aphids of random ages were infested onto 4 weeks old Arabidopsis plants which were then covered individually in cages as shown in Fig6. Gene transcript levels were evaluated after 5 defined time

intervals: 0, 1, 6, 48 and 72 hours. RNA extraction was carried out using RNeasy Plant mini kit (Qiagen) after the aphids had been gently removed using a fine brush. Aphid free plants were also brushed lightly with the brush to ensure equal treatment of all samples.



**Figure 6:** Individually caged Arabidopsis plants (Col-0) infested with GPA

**Data Analysis:** Normalized gene expression will be calculated using  $2^{-\Delta C_t}$  method (Livak and Schmittgen, 2001). Independent sample *t*-test ( $\alpha=0.05$ ) will be used to compare gene expression between WT Arabidopsis and the mutant genotypes.

### 3.4 Evaluation of the 3646 mutant's growth and development

Four week old GPA-infested and uninfested 3646 and WT Ws were used with 10 replicates for each treatment. For the infested plants, 30 aphids of random ages were placed on each plant for 1 week after which they were removed and allowed to grow. The plants were allowed to grow until they had fully matured rosettes. When the flowering stem was about 10cm high, an ARACON base was placed over the rosette leaving at least 1cm between the rosette leaves and the bottom of the base. An ARACON tube was then placed over the plants after the flowering stem has grown 10-15cm out of the ARACON base. Silique number and main stem length was taken for each plant after every four days. Seeds were harvested after the seeds had set and plants had dried out completely.

**Data Analysis:** The determination of possible trade-off will be analyzed using Independent-samples *t*-test ( $\alpha=0.05$ ) between the WT and mutant genotypes.

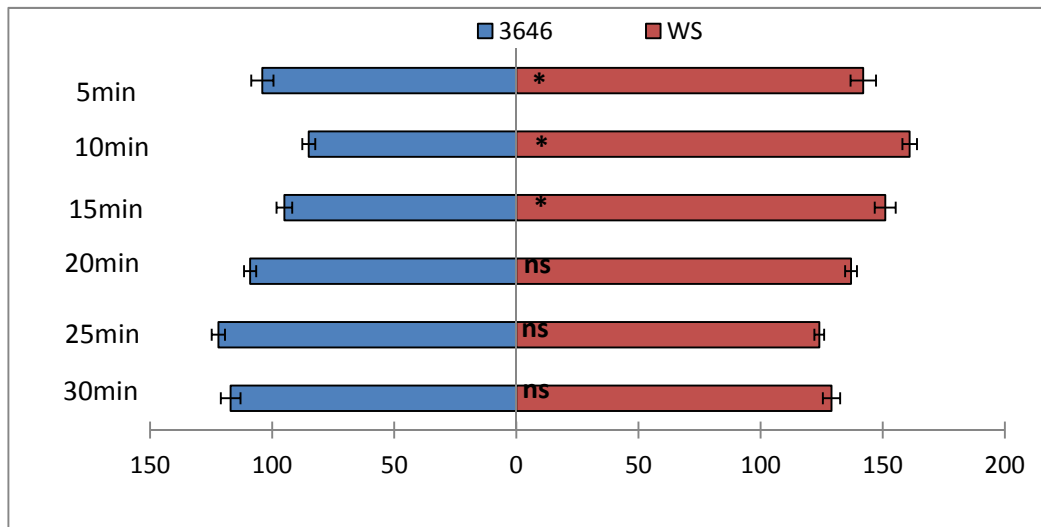
## 4.0 Results

### 4.1 Host Selection and Suitability Studies of Activation Tag Mutant 3646

Dual choice tests were carried out to evaluate the settling preferences of the GPA on either WT Ws or the Arabidopsis activation tag mutant 3646 using the cylinder setup at time points: 5min, 10min, 15min, 20min, 25min, 30min. Approximately 30 aphids per cylinder and 8 replications per genotype were used and the total aphid number per genotype for each time point was recorded (Fig 7). In this setup, the GPA were not in contact with the treatment plants but rather settling behaviour was influenced by the volatiles being constitutively released by the plants. Significant differences between the two genotypes were seen during the first 15minutes (5min:  $P = 0.0154$ , 10min:  $P < 0.0001$ , 15min:  $P = 0.0004$ ) as analyzed using  $\chi^2$  test. At time points 20min, 25min and 30min, no significant differences in host preference was observed ( $P = 0.0742$ ,  $P = 0.899$ ,  $P = 0.444$ , respectively).

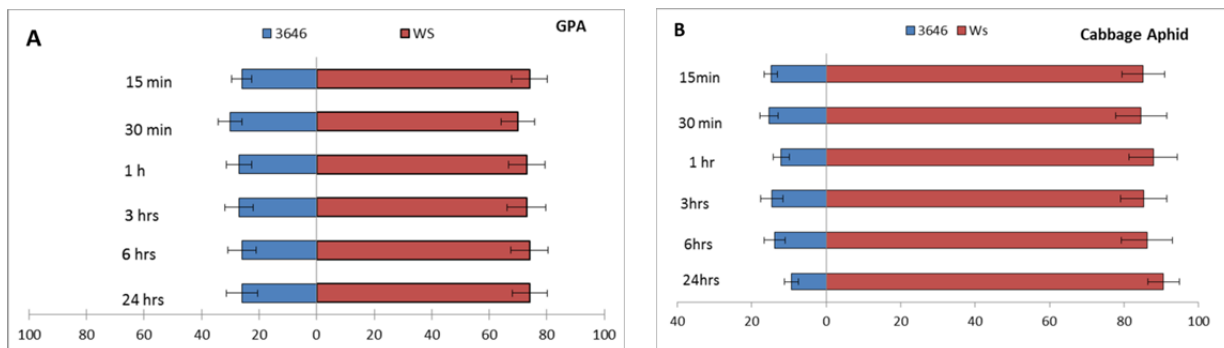
Dual choice tests using the pot setup to evaluate the settling behaviour of the generalist herbivore, GPA, on either WT Ws or the Arabidopsis activation tag mutant 3646 showed that a significantly high number of aphids were attracted to the WT than to the mutant plants.  $\chi^2$  test performed between the two genotypes at all the time points; 15min, 30min, 1hr, 6hrs and 24hrs, indicated significant differences ( $P < 0.000$ ). These results were similar to those obtained in the choice test involving the specialist herbivore, cabbage aphid ( $P < 0.0001$ ) where the aphids also showed a higher preference to the WT Ws than to the mutant (Fig 8).

A non-choice bioassay was performed to compare the two aphids' performance on the WT Ws and the 3646 mutant (Fig 9). Significant differences in the aphid numbers (adults and nymphs) found on WT Ws and 3646 were also noted ( $t = -8.459$ ,  $d.f = 32$ ,  $P < 0.0001$ ) thus confirming the results obtained in the dual choice tests. These results are consistent to previous observations of GPA resistance of 3646 as compared to its WT genotype (Chen et al., 2012)..



**Figure 7: Cylinder set-up dual choice test GPA on WT Ws and 3646**

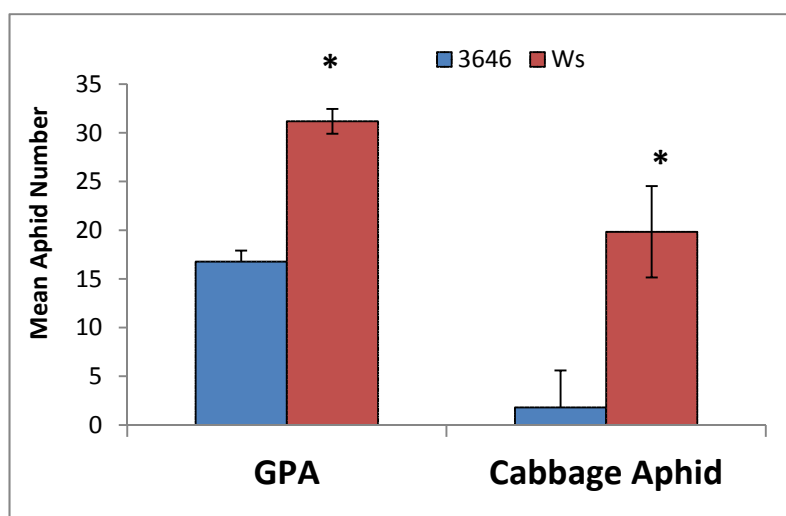
Host plant preference of approximately 30 aphids per cylinder (8 replications) was observed over 30min, every 5mins. Asterix (\*) indicate significant differences between the lines and (ns) no significant differences ( $p \leq 0.05$ ) for each type of aphid and the error bars represent SE generated at a confidence level of 95%.



**Figure 8: Host plant selection in a dual choice test of aphids between Arabidopsis activation tag mutant 3646 and WT Ws plants.**

The settling preferences of (A) 290 *Myzus persicae* (GPA) and (B) 256 *Brevicoryne brassicae*, (cabbage aphid) on either the activation tag mutant 3646 or WT Wassilewskija (Ws) Arabidopsis plants over six time intervals: 15min, 30 min, 1hr, 3hrs, 6hrs and 24hrs. Aphid responses are presented as a percentage of the number that chose a particular plant over the total number responding. The number of aphids that did not respond is also included on the right side of the bars.

§ Asterix (\*) indicate significant differences between the WT and mutant lines ( $p \leq 0.05$ ) with the error bars represent SE generated at a confidence level of 95%.

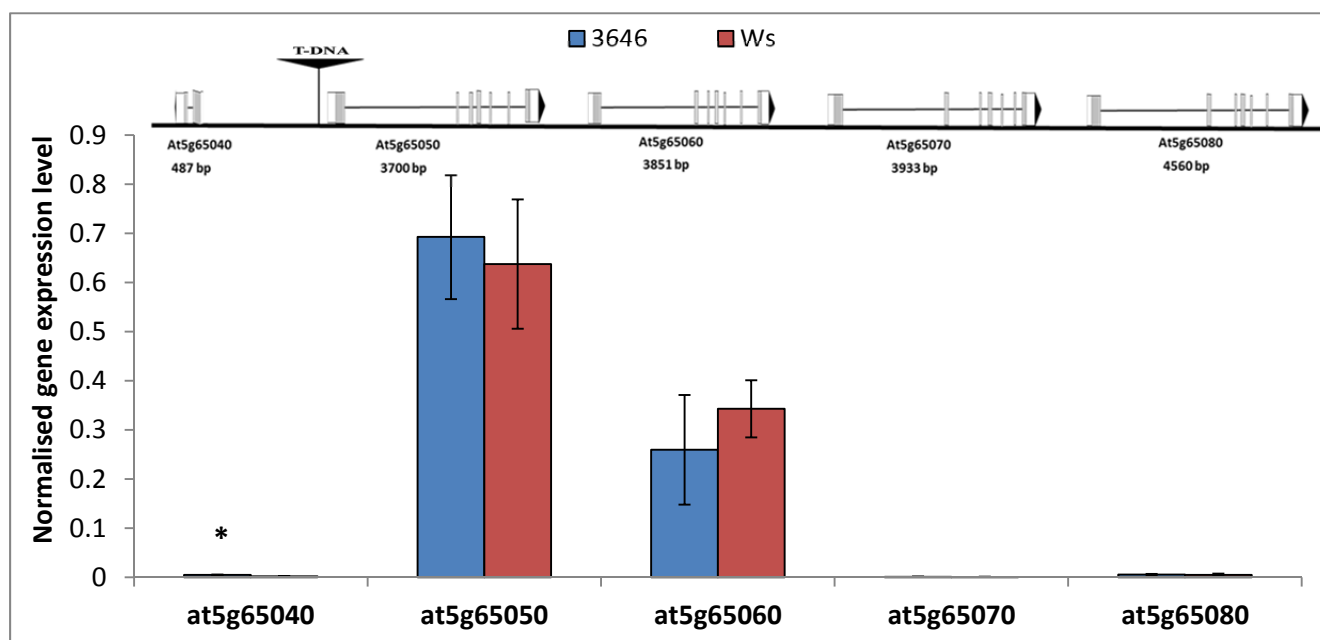


**Figure 9: Generalist GPA and specialist cabbage aphid non-choice tests between 3646 and WT Ws Arabidopsis lines**

GPA numbers recorded 14 days after release of one 1-day old GPA nymphs on each plant. Asterix (\*) indicate significant differences between the lines ( $p \leq 0.05$ ) for each type of aphid and the error bars represent SE generated at a confidence level of 95%.

## 4.2 Candidate Genes Expression Analysis of Activation mutant 3646

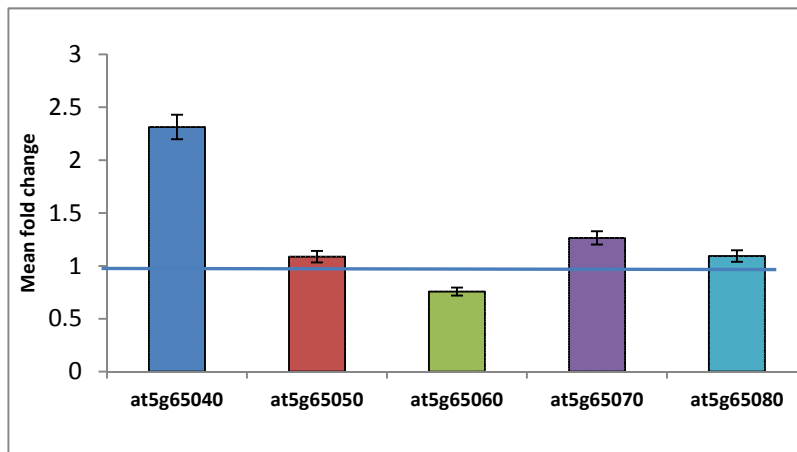
In order to determine which of the candidate genes flanking the T-DNA tag insert is involved in the aphid resistance in the mutant line 3646, gene expression level analysis was performed on both 3646 and WT Ws (Fig 10). Figure 11 clearly shows the high relative fold change of At5g65040 as compared to the rest of the candidate genes which had a 2.3 mean fold increase in the mutant relative to the Ws WT ( $t = 6.369$ , d.f. = 4,  $P = 0.000$ ).



**Figure 10: qRT-PCR gene expression levels analysis of the candidate genes in activation tag mutant 3646.**

Comparison of the expression levels of the candidate genes flanking the activation tag insert in 3646 compared against the WT Ws Arabidopsis line.





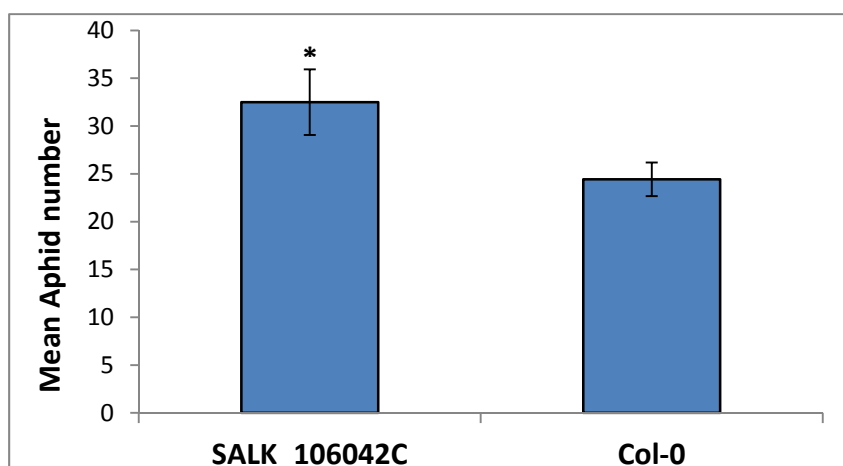
**Figure 11: Relative fold change of the candidate genes in 3646 mutant**

This was calculated as normalized gene expression of candidate gene in 3646 divided by expression in the WT Arabidopsis plant for each gene (WT set at 1). Horizontal line represents no change in expression.

### 4.3 Function Verification of At5g65040 Gene

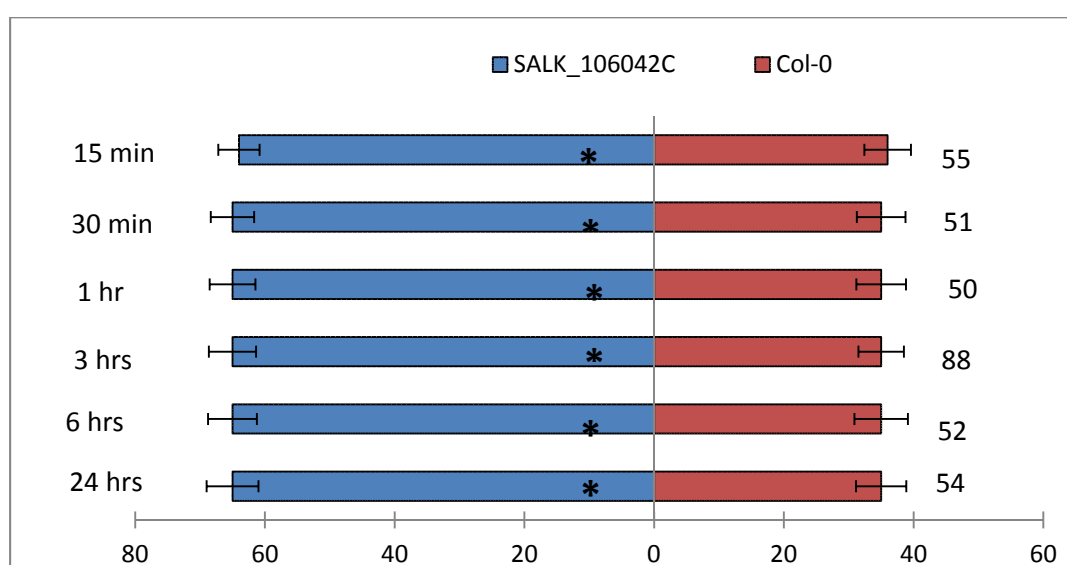
To evaluate the possible role of At5g65040 in Arabidopsis interaction with GPA, a non-choice aphid test was performed to make a comparison between the GPA performance on WT Col-0 plants and on At5g65040 knockout (KO) mutant (SALK line 106042C), which has a Col-0 background (Fig 12). The KO mutant has a T-DNA insertion within the coding region thus interfering with At5g65040 gene expression/function. A significantly large aphid population development on the Salk line was observed as compared to WT Col-0 ( $t = -2.005$ , d.f. = 28,  $P = 0.05$ ). To determine whether At5g65040 contributes to the antixenosis, a choice of either settling on the WT or the KO mutant was provided for the aphids using the pot set up (Fig 15). It was observed that the insects preferred Salk line106042C more than the WT Col-0.  $\chi^2$  test performed between the two genotypes at all the time points; 15min, 30min, 1hr, 6hrs and 24hrs, indicated significant differences ( $P < 0.0001$ ) thus confirming results observed in the non-choice test.

A GPA non-choice test carried out on an At5g65040 overexpression line in At5g65040 KO background (35S::40 (KO)) and compared against WT Col-0 and At5g65040 KO lines showed significant differences in aphid population development (ANOVA  $F = 2,102$ ;  $P = 0.0001$ ) (Fig 14). No significant differences between the overexpression line and the WT Col-0 were found (ANOVA  $F = 2,102$ ;  $P = 0.425$ ). In the overexpression line, the restored At5g65040 gene in the KO background resulted in the lowering of aphid number on the plants to the level of the WT Col-0 as compared to the significantly high aphid numbers on the line lacking At5g65040 expression (SALK 106042C).



**Figure 12: Non choice test of GPA on Col-0 and its At5g65040 knockout mutant.**

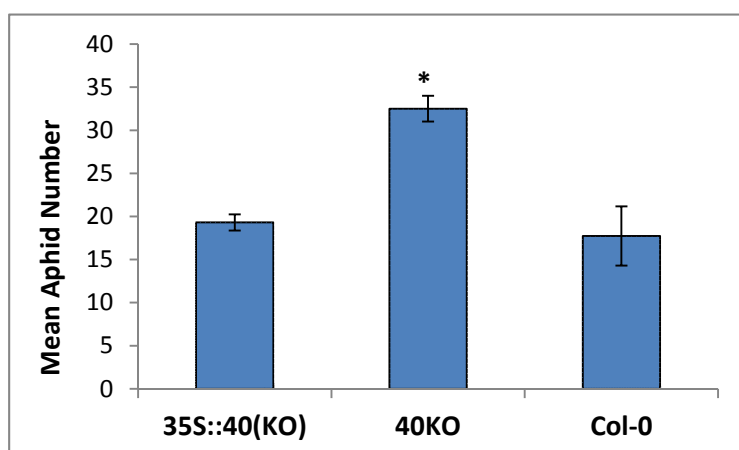
GPA numbers recorded 14 days after release of one 1day-old GPA nymphs on each plant. The mean was calculated from 15 replications per genotype. § Asterix (\*) indicate significant differences between the WT and mutant lines ( $p \leq 0.05$ ) with the error bars represent SE generated at a confidence level of 95%.



**Figure 13: GPA Host plant selection between Colombia (Col-0) At5g65040 gene knockout mutant line (SALK\_106042C) and its WT genotype**

Aphid responses of 300 GPA (30\*10 replicates) for each time point are presented as a percentage of the number that chose a particular plant over the total number responding. The number of aphids that did not respond is also included on the right side of the bars.

§ Asterix (\*) indicate significant differences between the WT and mutant lines ( $p \leq 0.05$ ) with the error bars represent SE generated at a confidence level of 95%.



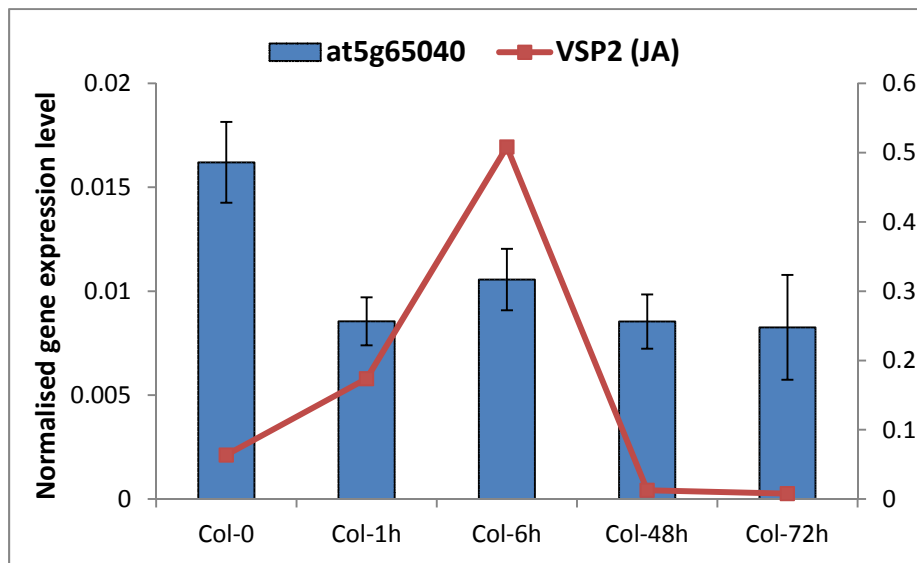
**Figure 14 Non- choice test including At5g65040 over expression line**

GPA mean numbers on 35S::40 in At5g65040 knockout line background, At5g65040 knockout line (40KO) SALK line 106042C and WT Col-0 Arabidopsis plants recorded 14 d.p.i by 1-day old GPA nymphs on each plant.

Asterix (\*) indicate significant differences between the lines ( $p \leq 0.05$ ) for each type of aphid and the error bars represent SE generated at a confidence level of 95%.

#### 4.4 At5g65040 gene expression pattern during GPA infestation

The At5g65040 gene expression in Col-0 infested with GPA shows that it is significantly down-regulated 1h.p.i whereas the well characterized JA-responsive gene, VSP2 is being upregulated (ANOVA  $F=4,10$ ;  $P\leq 0.01$ ) (Fig17). A significantly high expression of VSP2 was observed 6h.p.i (ANOVA  $F= 4,9$ ;  $P<0.001$ ) before returning to its initial level after 48 and 72h.p.i (ANOVA  $F= 4,9$ ;  $P>0.05$ ). Expression of the JA marker gene was used to indicate whether insect defence had been triggered by aphid infestation. Despite the steady up regulation of JA pathway, At5g65040 seemed to level-off its expression until the end of the experiment (72h.p.i).



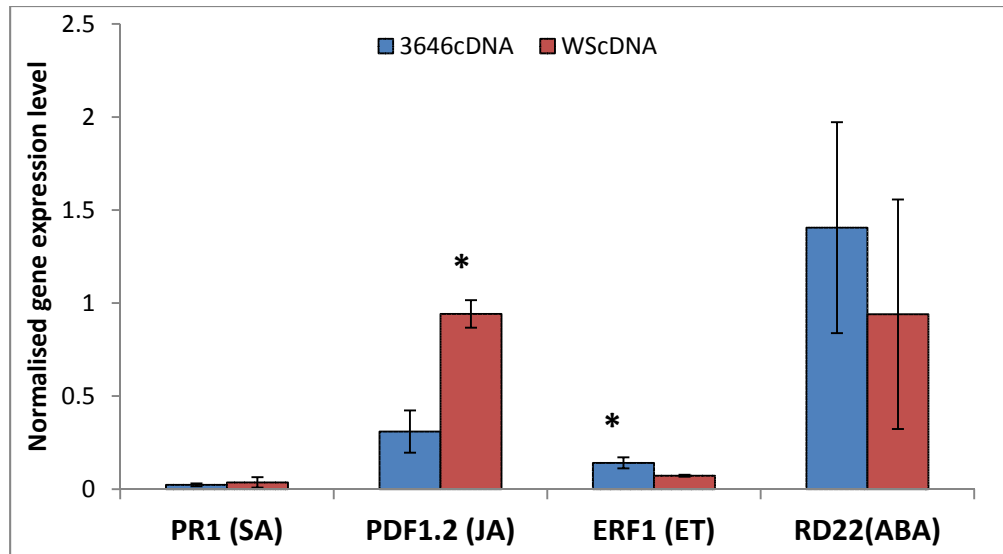
**Figure 15: GPA-induced JA marker gene, Vegetable storage protein (VSP2), and At5g65040 gene expression**

Gene expression evaluated on WT Col-0 Arabidopsis plants infested with GPA over different exposure times up to 72h time course. Three biological replicates with 17 plants pooled into each replicate were used to calculate gene expression of each treatment. The VSP2 gene expression graph indicates that insect defence has been triggered by aphid infestation in the plants. Error bars represent SE generated at a confidence level of 95%.

#### 4.5 Defence Signaling Pathways Marker Gene Expression Analysis in Activation mutant 3646

Gene expression analysis of defence signaling pathway marker genes showed no detectable changes in the SA and ABA signaling pathways ( $t = -0.799$ , d.f. = 4,  $P = 0.469$  and  $t = 1.165$ , d.f. = 4,  $P = 0.282$ , respectively) unlike the expression of ET and JA pathway marker genes, which were significantly altered in the mutant (Fig16). A down-regulation in the JA pathway was observed as indicated by a significant decrease in the expression level of its marker gene PDF1.2 ( $t=-8.087$ , d.f.=4,

P = 0.001). On the contrary, the ET pathway seemed to be up regulated significantly as seen in the increased expression of its marker gene ERF1 ( $t = 3.989$ , d.f = 4,  $P = 0.016$ ).



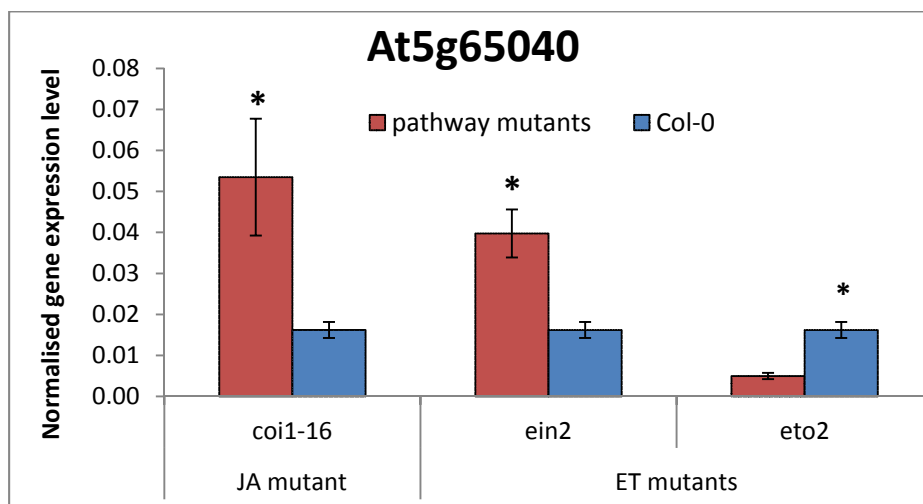
**Figure 16:** Gene expression levels of defence pathways marker genes

SA- (PR1), JA- (PDF1.2), ET- (ERF1) and ABA- (RD22) responsive genes as expressed in activation tag mutant 3646.

§ Asterix (\*) indicate significant differences between the lines ( $p \leq 0.05$ ) and the error bars represent SE generated at a confidence level of 95%.

#### 4.6 Gene expression of At5g65040 in signaling pathway mutants

At5g65040 expression in defective signaling pathway mutant lines of ET (ein2 and eto2) and JA (coi1-16) was significantly increased in coi1-16 and ein2 mutants ( $t = -4.492$ , d.f = 4;  $P = 0.011$  and  $t = -6.615$ , d.f = 4;  $P = 0.003$ , respectively). On the contrary, a significant down regulation of At5g65040 is noted in eto2 ( $t = 7.458$ , d.f = 4;  $P = 0.005$ ) (Fig 17).

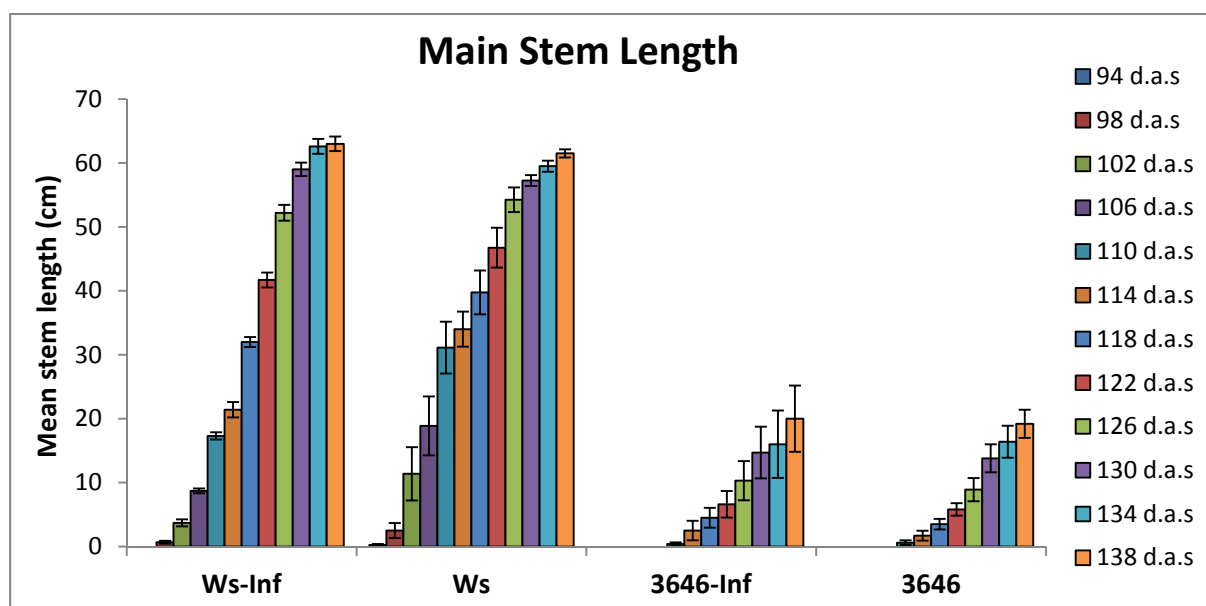


**Figure 17: qRT-QPCR gene expression analysis of At5g65040 in JA and ET defence signal pathway mutants.**

Error bars represent SE generated at a confidence level of 95%. § Asterix (\*) indicate significant differences between the WT Col-0 and mutant lines ( $p \leq 0.05$ ) and the error bars represent SE generated at a confidence level of 95%.

## 4.7 Trade-Off Evaluation of Aphid Resistance of 3646 at the Expense of Growth and Development

Growth parameters including main stem length (cm) and silique (seed pod) number were compared between 3646 and WT Ws Arabidopsis lines, GPA infested and uninfested with GPA. Significant differences were noted in the stem length among the four treatments (ANOVA  $F = 7, 38$ ;  $P = 0.000$ ) (Fig 18). Comparison between stem growth of the infested and uninfested WT Ws showed significant differences until 122 d.a.s ( $P < 0.01$ ) with more rapid growth being noted in the uninfested plants. Contrary to this, no significant differences were observed in stem growth of the infested and uninfested 3646 mutant plants throughout the whole experiment ( $P > 0.05$ ). However overall stem growth of the 3646 mutant plants was slower than in the WT Ws plants. At 138 d.a.s, the mean stem length of 3646 plants was 20cm and 62cm for the WT Ws plants.

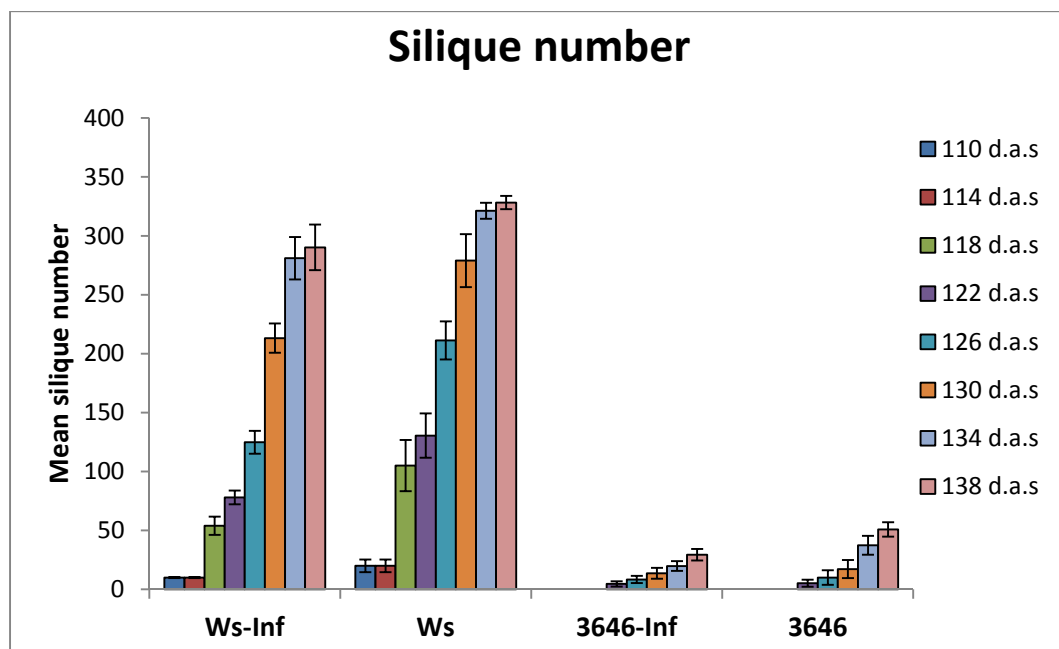


**Figure 18: Main stem length of GPA-infested and uninfested WT Ws and 3646 Arabidopsis plants**

Stem length was calculated as the average of 5 replications per treatment for each time period, given in cm. Error bars represent SE generated at a confidence level of 95%.

The mean silique number of the four treatments were significantly different with more siliques being produced by the WT Ws plants, both infested and uninfested (ANOVA  $F = 7.38$ ;  $P = 0.000$ ) (Fig 19).

Silques were produced 110 d.a.s and 122 d.a.s in the WT Ws plants and 3646 mutant plants respectively. Significant differences between the GPA-infested and uninfested WT Ws plants in silique production was observed throughout the duration of the experiment (138 d.a.s) ( $P < 0.01$ ). GPA infestation did not seem to affect reproduction of the mutant as no significant differences were noted between the two 3646 treatments ( $P > 0.05$ ).



**Figure 19: Silique number of GPA-infested and uninfested WT Ws and 3646 Arabidopsis plants**

The silique numbers are represented as means of 5 replications per treatment for each time period. Error bars represent SE generated at a confidence level of 95%.

The mutant was better able to tolerate aphid infestation than the WT Arabidopsis as shown by growth patterns of the stem and silique productions which had no significant difference (Fig 18 and Fig 19).

## 5.0 Discussion

### 5.1 Aphid Resistance of 3646 Mutant Line Is Based on Antibiotic and Antixenotic Mechanisms

In the dual choice test carried out in this study where aphids were not in contact with the plants, antixenotic effects of the 3646 mutant line were suggested. Constitutively released plant volatiles may possibly be contributing factors of antixenosis in this mutant during the first 15 minutes only as suggested by the results obtained in the cylinder- setup choice test. In another dual choice test carried out in this study in which the aphids were allowed to make their host choices whilst they were in contact with the plants supported the evidence obtained in the former set up; however, suggesting other antixenotic mechanisms besides the deterrent and/or attractant volatiles. I hypothesise the action of toxic and/or repellent allelochemicals or secondary metabolites against aphid infestation. The non-choice test carried out on the two aphids strongly suggested antibiotic effects of the mutant line as GPA population development was significantly lower on 3646 mutant than on the WT Ws plants. Toxic secondary metabolites affecting survivorship and fecundity, antidiigestive proteins e.g. PI which may hinder growth, can be implicated to these results (Simmonds, 2001; Tran et al., 1997)

*Brassica* species are known to constitutively produce GS (Louis J Fau - Singh et al.; Thompson and Goggin, 2006). Glucosinolates have been implicated to affect host preference of both generalist GPA and specialist cabbage aphid (Kim Jh Fau - Lee et al.; Levy M Fau - Wang et al.; Mewis et al., 2006; Rask et al., 2000). In another study, the fecundity of both aphids was higher on mutants producing lower levels of GS than on WT plants (Mewis et al., 2006). Upon tissue damage by aphid feeding, GS are hydrolysed by myrosinases resulting in the release of toxic insecticidal breakdown products which include the volatile and pungent isothiocyanates (Louis J Fau - Singh et al.; Rask et al., 2000). Isothiocyanates have been reported to be negatively correlated to GPA aphid numbers (Mewis et al., 2006). However, unlike the generalist GPA, specialist cabbage aphid has not been reported to be affected by GS breakdown products and moreover the effects of specific GS varies between the two aphids seems to vary (Cole, 1997). The cabbage aphid absorbs sinigrin GS into its haemolymph which is then hydrolysed by its own myrosinases to produce allyl-isothiocyanates which they use as a defence against its natural enemies.

Besides GS, antibiosis and antixenosis in *Arabidopsis* against GPA has been implicated in several publications on flavonoids (Simmonds, 2001). These compounds are known to modify feeding and oviposition behaviour (Harborne and Williams, 2000) and phlorizin has been reported to be an important feeding deterrent. However, flavonoids are not normally found in high concentrations in

phloem and moreover those involved in phloem transport are usually in inactive forms of their glycosides e.g. flavanone (Dreyer and Jones, 1981). Anti-feedants like PI proteins have been reported not to have much effect on phloem feeders as their diet contains free amino acids (Voelckel C Fau - Weisser et al.) Contrary to this, PIs have been found to affect fecundity, survivorship and weight in cereal aphid species in several studies (Tran et al., 1997). I therefore suggest chemical analyses of the leaf sap, including the phloem sap, for toxic or deterrent secondary metabolites. It is also imperative to carry out gas chromatography mass spectrometry (GCMS) tests in which the full spectrum of the volatile metabolites making up the volatile plume in the headspaces of the WT and mutant lines to help affirm these hypotheses.

Mechanical defences cannot be ruled out as the presence of leaf surface glandular trichomes or modified cell walls could be contributing factors to host preference. Callose depositions triggered by stylet damage and phloem protein coagulation at the sieve elements are common defences affecting sap availability and subsequently deterring the aphids (Diaz-Montano J Fau - Reese et al.). Studies made on the cabbage aphid have revealed the increased expression of callose synthase gene (*CALSI*; At1g05570) (Kusnierczyk A Fau - Winge et al.) thus suggesting that aphid infestation stimulates callose biosynthesis.

Induced changes in the cell wall structure have been shown in studies of cabbage aphid and GPA infestation on Arabidopsis where Xyloglucan endotransglycosylase (XTH) and pectin methyl esterases were upregulated (Divol F Fau - Vilaine et al.; Kusnierczyk A Fau - Winge et al.; Moran and Thompson, 2001) . XTH modifies hemicelluloses to confer strength to the cell walls whilst methyl esterases control pectin assembly for a more stiffer/ rigid structure (Peña et al., 2004). **The** expression of XTH33 (At5g02310) was shown to be altered after aphid feeding and higher populations were found on xth33 mutants suggesting that XTH33 is involved in providing cell wall mediated defence against GPA (Divol F Fau - Vilaine et al.). However its involvement in aphid control has been suggested to be working in conjunction with other gene(s) in Arabidopsis (Louis J Fau - Singh et al.).

These factors could contribute to the antixenotic properties of the mutant as they affect the ability of the aphid to reach the sieve elements (Louis J Fau - Singh et al.). Phloem proteins which coagulate after stylet penetration also have a bearing on the time the aphid will maintain its feeding site (Will and van Bel, 2008). Using the Electrical Penetration Graph (EPG), the feeding patterns of aphids can be characterised in order to have a detailed study of plant resistance to the phloem feeders at the pathway, phloem and xylem phases. (Diaz-Montano J Fau - Reese et al.; Pegadaraju et al., 2007; Tjallingii, 2006). Supporting chemical analyses of the phloem sap could give more conclusive results on what chemical defences are being employed by the mutant at each phase.



## **5.2 At5g65040 Gene is Involved in GPA Resistance in Activation Tag Mutant 3646**

I present At5g65040 to be a possible gene flanking the insert tag showing significant difference between the WT Ws and the GPA resistant mutant line with a fold change of 2.3. None of the MAF box genes seem to be involved. Function verification of this gene was proved in the dual choice test where a higher preference of GPA for SALK line 106042C as compared to the WT Col-0 was observed. In the absence of this gene, GPA thrived to affirm the possibility of antibiotic influence of At5g65040 in the non-choice test. The replacement of the knocked out At5g65040 gene in the 35S::40KO mutant line to give a lower aphid population development, further confirmed the role of At5g65040 in aphid control in this mutant. I recommend similar functional studies using over expression lines of At5g65040 in a Ws background in order to present more conclusive results.

An evaluation of the transcriptional changes of At5g65040 in response to GPA infestation suggested that GPA may be down regulating its expression as a mechanism to avoid plant resistance. Consequently, the need to study how the gene is expressed in 3646 during aphid infestation cannot be overstated. According to The Arabidopsis Information Resource (TAIR), At5g65040 is expressed at different stages of growth and development and in all plant tissues. Not much information is available on the transcript levels at each stage or in each tissue. In this study, whole plants were used to assess expression of the gene and was found to be very low in both the mutant and the Ws plants as compared to the Col-0 plants.

The constitutive expression of At5g65040 in both 3646 and the over expression line could be working against the ability of the aphid to lower the levels of its product.

## **5.3 Ethylene Pathway Is Involved In GPA Defence Response In Activation Tag Mutant 3646**

Phloem feeding insects, specifically aphids and whiteflies, have been shown to induce the expression of SA- and wound responsive JA regulated genes thus suggesting their role in PFI defence (Moran and Thompson, 2001). Despite the evidence of an antagonistic interaction between the two pathways (Kessler and Baldwin, 2001), microarray studies on the transcriptional responses of aphid-Arabidopsis interactions have revealed a significantly greater expression of transcriptional signatures of SA signal pathway ( $\beta$ -1-3-glucanase and PR1) as compared to those of the JA/ethylene (antimicrobial defensin PDF1.2) and of the major insect response/wound pathways (PI and LOX2). Of the two pathways, SA signalling is the most important in phloem feeders as little damage is inflicted by the stealthy insects during probing or stylet penetration (Walling, 2008; Zhu-Salzman et al., 2004). In this study, however,

the expression of SA- responsive marker gene (PR1) was not altered in the mutant 3646. JA was down-regulated whilst the ET pathway was significantly upregulated.

At this point of our study, it cannot be established yet which signalling pathway is involved during aphid infestation and thus in resistance. I therefore recommend gene expression analyses of the signal pathway marker genes in aphid infested 3646 mutant plants. Whether the ET would remain upregulated after infestation will be established in these further studies.

The expression levels of At5g65040 in the ET and JA pathway mutants; *eto2* and *coil-16* respectively, indicate a differential regulation between At5g65040 and JA and ET pathways. When the ET pathway was degenerate (*eto2*), At5g65040 expression was significantly low and was highly expressed when JA pathway was defective (*coil-16*). The *coil* (At2g39940) gene is required for a plant's responsiveness to jasmonates thus leading to defence regulation (TAIR). It is an F-box component of E3-ubiquitin ligase, SCF<sup>COI1</sup> involved in the derepression of JAZ repressor proteins for the regulation of the wound and jasmonates-induced defence (Howe and Jander, 2008). The up-regulation of At5g65040 in the jasmonates-insensitive *coil-16* could possibly suggest an antagonistic interaction. However, it is too early to conclude on this hypothesis with only these results.

The *eto2* mutant is defective in its ability to produce aminocyclopropane-1-carboxylic acid (ACC). ETO2; Ethylene Overexpression 2 (ACC synthase 5-ACS5) is one of the six isoforms of ACS genes, identified as ACS1-ACS6 (Liang et al., 1992; Van der Straeten et al., 1992) involved in ethylene (C<sub>2</sub>H<sub>4</sub>) production in response to low concentrations of cytokinin in etiolated seedlings (Vogel et al., 1998). An enzyme called ACC oxidase catalyses the conversion of ACC into ethylene as the final stage of ethylene biosynthesis (Woeste et al., 1999). Interestingly, in another defective ET pathway mutant, *ein2*, transcription of At5g65040 was upregulated in contrast to what was observed in *eto2*. EIN2 (At5g03280); Ethylene Insensitive 2 is an NRAMP metal transporter acting downstream of ETO2 (Chen et al., 2005) and ETR2 (ET membrane receptor) (Buer et al., 2006) in the ET biosynthesis pathway. If At5g65040 is down-regulated after ETO2 knockout and increased after the expression of ACC synthase gene is suppressed, it seems probable that At5g65040 acts upstream of EIN2 and downstream of ETO2. Thus said, I present a hypothesis that its expression could be regulated with ethylene. To test this hypothesis may require transcription level analysis of At5g65040 after exogenous applications of ethylene on WT and *eto2* mutants. Mutants *eto1* and *eto3* overproducing ethylene could also be used in these experiments to evaluate At5g65040 gene expression.

It is also imperative to test whether there could be antagonistic or synergistic relationships between At5g65040 and the JA and ET pathways by evaluating gene expression studies involving overexpression and knockout lines of both At5g65040 and JA/ET signal pathway lines.

## 5.4 Cost of Defence

Certain traits in plants that confer insect resistance may be expressed constitutively regardless of herbivore presence, or may be induced after infestation. The latter mode of defence regulation ensures lower costs of resource allocation being incurred thus minimising fitness consequences (Howe and Jander, 2008; Kessler and Baldwin, 2001). In 3646, normal growth and development was severely compromised. GPA infestation did not seem to affect reproduction of the mutant as no significant differences were noted between the two 3646 treatments implying no compromise in reproduction by defense mechanisms in the mutant.

Whether fitness-limiting resources e.g. Nitrogen, are reallocated towards defence, or toxic secondary metabolites detrimental to the plant's metabolism are involved, cannot be established at this point. Despite its proved resistance against aphids, the poor growth traits accompanying resistance may not be practically useful in crop production. However, identification of At5g65040 gene implicated in this study as the most probable gene involved in aphid resistance, provides a step towards improving susceptible lines which have favourable growth traits using conventional breeding methods. Over expressing the gene in the susceptible plants is also a possible way of breeding for aphid resistance cultivars. Fitness effects, however, of incorporating At5g65040 into the cultivar will have to be evaluated.

## Conclusions

This study has identified At5g65040 as a gene involved in the aphid- Arabidopsis interaction. Despite its unknown biological function, its antixenotic and antibiotic effects against both specialist and generalist aphids have been evident. However, specific elicitors and defence metabolites involved in this system have not yet been established. Thus said, EPG and microarray studies on the analysis of 3646-aphid interaction may be important tools to further elaborate the mode of defence employed against aphids. Despite the fact that the ET signalling pathway is up regulated in this mutant, its role in aphid resistance cannot be concluded at this point.

As the mutant of our study has been generated from Ws ecotype, I propose the use of mutants with a similar background in continuing studies.

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## Appendices

### Appendix I : Candidate Genes and Signal Pathway Marker Genes Primer Sequences

Gene	Forward Primer sequence	Reverse Primer Sequence
<b>At5g65040</b>	5'-TCTGCCATCATCGTGACATT-3'	5'-TTTGCTTCTCCCTGCATTCT-3'
<b>At5g65050</b>	5'-GGAATGTCATGGGAAAATGG-3'	5'-AGCTCAGCCGTTGATGATG-3'
<b>At5g65060</b>	5'-AGGAATGTCACGGGAAAATG-3'	5'-AAAGCTCAGCCGTTGATGAT-3'
<b>At5g65070</b>	5'-CGAGCTTGTGAAGACCCATC-3'	5'-CTCTTGCATCCTCTGCTTCC-3'
<b>At5g65080</b>	5'-GTGAAGCCATGGGAAGAAGA-3'	5'-CGAGCTTCTCCATGAGACC-3'
<b>PR1</b>	5'-GGCCTTACGGGGAAAACCTTA-3'	5'-CTCGCTAACCCACATGTTCA-3'
<b>PAD4</b>	5'-GTTCTTTTCCCCGGCTTATC-3'	5'-CGGTTATCACCACCAGCTTT-3'
<b>PDF1.2</b>	5'-CACCTTATCTTCGCTGCTC-3'	5'-GCACAATTCTGTGCTTCCA-3'
<b>LOX2</b>	5'-AGATTCAAAGGCAAGCTCCA-3'	5'-ACAACACCAGCTCCAGCTCT-3'
<b>VSP2(At5g24770)</b>	5'-TACGAACGAAGCCGAAGTCT-3'	5'-GGCACCCTGTGCAATCTAT-3'
<b>ERF1</b>	5'-CTTCCGACGAAGATCGTAGC-3'	5'-TCTTGACCGGAACAGAATCC-3'
<b>ORA59(At1g06160)</b>	5'-GGCTCTCGCTTATGATCAGG-3'	5'-TCCGGAGAGATTCTTCAACG-3'
<b>RD22 (At5g25610)</b>	5'-CCGACGTAGGAGTCGGTAAA-3'	5'-ATCGTCGTGGAGCTGAGTCT-3'
<b>ADH1.4(At1g77120)</b>	5'-TTCAACAGGTGATCGCTGAG-3'	5'-CAACCATCGTGACACATTC-3'
<b>LSH7( At1g78815)</b>	5'-AAGCGTGGGGGAGTTTAGAT-3'	5'-CCTTCGCCTGTGAATCTCTC-3'
<b>FLS2(At5g63580)</b>	5'-AATGGCTGAGCAATGGAAAG-3'	5'-GGTCCGATAACCACATCAGG-3'

Source: <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>

### Appendix II: 3 Primer Sequences For SALK Line Homozygosity Tests

Salk line	Knocked out gene	Left genomic primer (LP)		Right genomic primer (RP)	
<b>SALK_053639.53.20.x</b>	At5g65040	C048	TAAATGTCACGATGATGGCAG	C049	TCCGCACTAACTTTTGTCATG
<b>SALK_106042.49.15.x</b>	At5g65040	C050	CACGAACAAATCAAATCATGC	C051	TGAAAATTTGAATCACTGGTTG
<b>SALK_085852.24.85.x</b>	At5g65080	C052	CGTTTACGTCTCTTGAAACG	C053	TGGGTTTCACCAGAAAGTGTG
<b>SALK_044822.21.20.x</b>	At5g65060	C054	CGAGCGGAATATCGTCATATG	C055	AAAAATCTTGAATCGCATGC
<b>SALK_028506.33.90.n</b>	At5g65070	C056	TGATTACATATGCATGCGACC	C057	TGAGTTATTGGGTCTCATGGG
<b>SALK_141778.47.75.x</b>	At5g65050	C016	TGTGAGTACAATGAACCAAAACG	C017	GAGAAAACGAAGGGAATGGTC
<b>LB1.3 (left border primer of the T-DNA)</b>		C005	ATTTTGCCGATTTTCGGAAC		



## Appendix III

### PCR PROTOCOL

1. For a 25ul rxn:

- Use 1ul of 60ng/ul or 100ng/ul DNA
- Use 1ul of each primer at 3.2pmole/ul concentration or 1.25ul of each primer at 100ng/ul concentration
- 2.5ul 10x PCR Buffer w/ Mg (1.5mM)
- 0.5ul 25mM MgCl<sub>2</sub>
- 0.5ul dNTP
- 0.125ul Taq
- 18.375ul sterile water to equal a 25ul rxn

(\*if not making master mix, dilute Taq so that you can add 1ul of Taq and 17.5ul sterile water to equal a 25ul rxn)

2. Keep the reagents on ice.

3. Add the Taq last, and keep it in the freezer until you are ready to add it.

4. Vortex briefly and quick spin.

5. Cycle:

- 95°C for 1-5minutes (usually 4min)
  - 95°C for 1min
  - 55°C for 1min
  - 72°C for 1.5 to 2min (usually 2min)
  - 72°C for 10min
  - 4°C hold
- Cycle 30 times