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Using Reverse Genetics in Screening for Host Resistance of Arabidopsis Plants to Small Cabbage White Butterfly*Pierisrapae*Herbivory

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ABSTRACT.

Small cabbage white butterfly *Pierisrapae* is one of the most destructive specialist herbivores of the *Brassicaceae* plants. Chemical application has been used for a long time to control *P. rapae* but this has been shown to have major draw backs. Owing to their disadvantages, we investigate in this study, the host plant resistance as an alternative control measure for specialist insect *P. rapae*. T-DNA mutant *Arabidopsis* plants were screened for their variation in response to *P. rapae*.None choice and two-choice test were conducted on 30 and 2mutants respectively. For the nonechoice test, four mutantsshowed a difference response in comparison to the wild type as measured by biomass reduction. These mutants were disrupted in AT1G09920 (TRAF-TYPE ZINC FINGER-RELATED), AT1G79460 (ENT-KAURENE SYNTHASE 1), AT1G10070 (BRANCHED-CHAIN AMINO ACID TRANSFERASE 2) and AT1G10090 (EARLY-RESPONSIVE TO DEHYDRATION 4). For the two-choice test, one mutant showed a difference response in comparison to the wild type as measured by larvae preference. This was disrupted in AT2G24210 (TERPENE SYNTHASE 10). This study concludes by recommending validation experiment and research to uncover

the molecular basis of these findings and then transfer these novel genes to crop plants.

Keywords:*Arabidopsis thaliana, Pierisrapae*, Host plant resistance, Mutant, Genes.

1 INTRODUCTION.

Small cabbage white butterfly *Pierisrapae L* (Lepidoptera: Pieridae) is one of the most destructive specialistherbivore of the *Brassicaceae* plants(Agrawal & Kurashige, 2003). *Brassicaceae*family include important crop to our diet such as the oil seeds (e.g., canola and mustard), cole crops (e.g., cabbage and cauliflower) and root vegetables (e.g., radish and turnip). Owing to their high dietary value, their scale of production has increased dramatically over the past few decades(Furlong et al., 2013).This increase has led to pressure on land and modification of the landscape. Consequently, the brassica vegetables are now being grown in areas where they were not originally cultivated and this led to a renewed challenge in controlling pest such as the*P.rapae*(Furlong et al., 2013). Plants within the *Brassicaceae* family contain a diverse phytochemicals such as glucosinolatesfor defence purposes against most herbivores. However, specialist insect, *P. rapae,* feeds on them and seemingly unaffected by glucosinolates and proteinase inhibitors(Agrawal & Kurashige, 2003). It has developedstrategies to circumvent these toxic compounds(Ratzka et al., 2002).

Synthetic pesticides were originally dependent on as a pest management strategy to a wide range of insects. Their use grew exponentially to a near-exclusive control measure until a multiple side effects such as outbreaks of secondary pests, insect developed resistance and destruction of beneficial insects leading to resurgence of targeted pest population became apparent (Kennedy, 2008). This led to adoption of integrated pest management approach which focuses on a combination of biological, cultural, host plant resistance and as a last option chemical use(Tabashnik et al., 2008).

Host plant resistance traits.

Host plants resistant traits relies on the plants ability to tolerate or resist myriad of attacks from herbivores(Howe & Jander, 2008).These traits can be based on

antixenosis or antibiosis. In Antixenosis, the plants emit deterrent volatiles that reprogram the plant inclination to the host (Gibson & Pickett, 1983). In antibiosis, the reproduction and population development of the insect pest that made contact with the plant is reduced(Smith & Boyko, 2007).

Plants rapidly detect intruders and triggers immune systems that help exclude the intruders. The immune system is mediated through two branches; PAMP-triggered immunity (PTI) and Effector-triggered immunity (ETI) (Dangl et al., 2013; Erb et al., 2012;Jones & Dangl, 2006). PTI involves the transmembrane patterns recognition receptors (PRRs). These receptors respond to evolutionary conserved molecules such as Microbe, Pathogen, Damage and Herbivore associated molecular patterns (MAMPS/PAMPS/DAMPS/HAMPS)(Dangl et al., 2013; Erb et al., 2012;Jones & Dangl, 2006). These molecular patterns activate the PRRs and this leads to intracellular signalling, transcriptional reprogramming and biosynthesis of complex compounds that restricts microbial establishment (Karban et al., 1997). The PTI and ETI are regulated by the phytohormones which are produced in the cells in extremely low quantities and the production depends on the strategies and the lifestyle of the attacker (Pieterse et al., 2009). There are two major defence plant phytohormones: salicylic (SA) and jasmonic acid (JA) with its derivatives called jasmonates(Pieterse et al., 2012).However, studies have shown there are other hormones such as ethylene (ET), abscisic acid (ABA), gibberellins (GAs), auxins, cytokinins (CKs), brassinosteroids, and nitric oxide (NO) also play a critical role in regulating plant immune signalling network(Pieterse et al., 2012). The hormonal immune network can either interact synergistically or antagonistically to achieve optimal plant defence response(Robert-Seilaniantz et al., 2011). SA and JA are known to antagonize each other and in this antagonism, the plants prioritize one pathway over the other depending on relative concentration of each hormones, timing and sequence of attack(Pieterse et al., 2009).

When the pathogen effectors overcome the plant resistance genes, it isimperative to find new heritable genes that confer resistance to the pathogens in question. In *Brassicaceae*crops this can be investigated using a model plant *Arabidopsis thaliana* (L.) Heynh(Feng & Mundy, 2006). *Arabidopsis* grows in diverse climatic, edaphic and altitudinal habitats that expose it to a wide range of selection pressure. This gives rise

to genomic diversity across geographical distribution resulting to a broad phenotypic variety and local adaptation. This natural variability provides additional resource for the study of molecular and ecological functions of genes (Chao et al., 2012). To link a gene to a phenotypic diversity, a Genome Wide Association techniques is used among other techniques (Nordborg & Weigel, 2008).

Once the genes have been identified, forward and reverse genetic tools can be used to elucidate their functions. Forward genetic tools relies onnatural or artificial induced mutations that give rise to a mutated phenotype followed by cloning the corresponding genes (Feng & Mundy, 2006). While reverse genetics relies on the mutation of gene of interest and the resulting phenotype observed to predict gene function (Tierney & Lamour, 2005). The genes can be mutated using techniques such as T-DNA and transposon mutagenesis, chemical mutagenesis and site directed mutagenesis(Feng & Mundy, 2006).T-DNA has been used to mutate many genes in *Arabidopsis*and these mutants are stored in *Arabidopsis* stock centres (http://arabidopsis.org).

This work follows a previous study in which a GWA experiment was curried out to determine the candidate genes for this study.The candidate genes were mutated using T-DNA insertion. The mutated genes were ordered form *Arabidopsis* stock centre (http://arabidopsis.info/). In this study we used reverse genetics tools to investigate the variation of response of mutated *Arabidopsis* plants to a specialist insect *P. rapae.*We use loss of function mutations and thus the mutated gene produce less protein or some functions of the proteins have been compromised.

2 MATERIALS AND METHODS:

2.1 Plant materials:

*Arabidopsis thaliana*Heynh ecotype Columbia (Col-0)wild type and a collection of 30 mutant seeds were obtained from the European Arabidopsis Stock Centre (NASC) (http://arabidopsis.info/). The plants were grown in 0.08 L growing pots filled with *Arabidopsis* growing media and 30 seeds were planted per mutant. The plants were grown in ashort day climate controlled chamber at 21±1°C, 50–70% RH, witha 8:16 L: D regime.

2.2 Genotyping.

After two weeks, the leaf samples were collected in an Eppendorf tubes and frozen in liquid nitrogen. For each mutant 10 samples were collected for the first screening. Successive screening was done until at least one homozygous mutant was identified. The leaves were grinded in liquid nitrogen to break the leaf tissues. The DNA was extracted using Simple genomic DNA isolation technique (Jacqueline Busscher), supplementary information 1 (SI 1).

Polymerase Chain Reaction(PCR) was used to screen for the plant genotype. This was done using the genomic specific primers and the T-DNA left border primer. Primers specific to T-DNA line, (ATTTTGCCGATTTCGGAAC for Salk line and GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC for Sail lines), were obtained from signal web resource (http://signal.salk.edu/). The reverse and forward primers for the wild type were designed and ordered. The amplification reaction were performed in 50 µl solution containing 0.2 µM of each primer, 0.2 µM of each dNTP, 5X Ampliqon reaction buffer with 15 µM MgCl2, and 1 U of Ampliqon DNA polymerase (Ampliqon IIII). For the amplification, thermo cycler was set as follows: 1 cycle at 95 °C for 3 min, 35 cycles at 95 °C for 30 sec, 60-55 °C for 1 min and 72 °C for 1.30 min and then 1 cycle at 72 °C for 5min followed by cooling to 4°C until the run is stopped manually.The specific primers used for each gene are described in SI 2.

The PCR products were separated on 1 % agarose gel, stained with ethidium bromide and visualized in UV light. The digital photographs of the fluorescent ethidium bromide-stained DNA separation patterns were taken (Fig 1). Heterozygous mutants'and wild type plants were discarded and the homozygous mutants'plants transferred to the long day chamber at temperature 21^0c, 70% relative humidity, and a diurnal cycle of 16-h day and 8-h night,to generate seeds for bioassays.

2.3 Insects:

The small cabbage white butterfly *P. rapae*were rearedon Brussels sprouts plants (*Brassicaoleraceae* var. gemmifera). Rearing took place in the glass houses at

21±1°C, RH of 50–70% diurnal cycle of 16-h day and 8-h night in Wageningen University and Research Centre.

2.4 None choice experiment:

The plants were propagated in the short day growing chamber as described above. After five weeks, the experiment was set up for each T-DNA mutants to assess the response of the mutant to *P. rapae.* The wild type Col-0 was used as a reference plant. Two treatments were used: infested (Pieris) and without infestation (control). On each genotype (mutant and wild type), the two treatments were applied with 20 replicates per treatment/genotype combination. The experiment was laid in a completely randomized design. Each plant was infested with one newly hatched L1 *P. rapae*using a soft paint brush directly onto the leaves. The plants were then transferred to plastic pots with a mesh lid for aeration to limit larvae movement. After seven days, the plant rosette weights were measured and recorded.

2.5 Two choice tests:

Three genetic lines were used for the choice test; Col-0 was compared separately to ***at2g24210*** and ***at3g11480***to assess the effect of mutation on *P. rapae* behaviour. Col-0 plant was compared with one mutant and replicated 13 times for ***at2g24210*** and 18 times for ***at3g11480***. Pair of plants were placed inside a small plastic container and a bridge made of filter paper placed in between them. Newly hatched L1 *P. rapae* larvae were starved for at least one hour in a plastic container and 10 caterpillars were placed one after another on the middle of each bridge using a paintbrush and the preference of the larvae scored. After the first preference test at a time point 0, each plant was infested with one larva to induce the plant defence. The test was then repeated after 72 hours.

2.6 Statistical Analysis

All statistical analyses were performed using GenStat 15 edition. A two-way ANOVA was performedto test for significant interaction between treatment and genotype on plant rosette weight. Differences were considered significant if the P value was ≤ 0.05 for the two tests. To test the effect of genotype on the choice of *P. rapae* larvae, a Chi-squire test was used to analyse whether the total number of larvae that chose for the

Col-0 plants and the total number of larvae that chose for the T-DNA mutant ***at2g24210*** or ***at3g11480***plants significantly differed from a 1:1 distribution (P<0.05).

3 RESULTS AND DISCUSSIONS

3.1 None choice bioassays.

A total of 30 mutants were screened (Table 1) and four mutants showed significant interaction between genotype and treatment implying that mutation of the gene influence biomass reduction in response to *P. rapae*herbivory. The T-DNA mutants that display a significant interaction (Two way ANOVA, p≤ 0.05)(Table 1) are:***at1g09920***, ***at1g10090***, ***at1g79460*** and ***at1g10070.***

The ***at1g09920***T-DNA mutant showed a lower reduction in biomass in two independent experiments in comparison to the wild type Col-0 (Two way ANOVA, p≤ 0.001 and p= 0.05) (Figure 3A&B).The Arabidopsis Information Resource (TAIR) describes the gene as TRAF, TYPE OF ZINC FINGER LINKED proteins. Zinc finger proteins are members of nucleic acid binding proteins in eukaryotes. They consist of comparatively small proteins motifs that have numerous finger-like protrusions that bind to specific targets. They are currently known to bind DNA, RNA, protein and/or lipid substrates depending on the amino acids sequence of the finger domain (Brown, 2005). In mice, TRAF-type of zinc finger domain protein 1 has been found to scale down innate immune responses. It negatively control the activation of Toll-like receptor 4 (TLR4) by directly reacting with it and lowers the activation of NF-kappa-B. It also suppresses excessive activation of DDX58/RIGI-like helicases (RLH) pathways (Sanada et al., 2008). Using this model from mice and gene homology, I may propose that Gene AT1G09920 may be involved in the negative regulation of the basal immune response in *Arabidopsis* plants and by mutating this gene; the plant may express fully its basal immune signalling pathway that leads to a resistant phenotype.

The ***at1g10090*** T-DNA showed higher and lower reduction of biomass in two independent experiments. In the first experiment, it showed a lower reduction in biomassthan the wild type Col-0 (Two way ANOVA, p= 0.038) (Figure 3C) while in the second experiment it showed a higher reduction in biomass than the wild type Col-0

(Two way ANOVA, p= 0.007) (Figure 3D). The function of gene AT1G10090 in the wild type is unknown. However, it's disrupting gave two contradictory results, therefore, we cannot be able to predict its exact contribution using the outcome of this study. However, I recommend more experiments to ascertain its exact contribution.

The ***at1g10070*** T-DNA mutant showed a higher reduction in biomass than the wild type Col-0 (Two way ANOVA, p=0.002) (Figure 3E). Gene ATIG10070 encodes chloroplast branched-chain amino acid aminotransferase (BCAAs) (//www.arabidopsis.org/). BCAAs biosynthesis is catalysed by a set of hormones with branch chain aminotransferase (BCAT) catalysing the final stage. (Binder, 2010). The BCAT enzymes are encoded by different genes in *Arabidopsis* such as BCAT1 (AT1G10060), BCAT2 (AT1G10070), BCAT3 (AT3G49680), BCAT4 (AT3G19710), BCAT5 (AT5G65780) and BCAT6 (AT1G50110). It has been shown that the expression levels of BCAT2 in the wild type correlates with the biosynthesis of BCAAs production (Binder, 2010). BCAAs and met chain elongation forms the start of the synthesis of vast spectra of glucosinolates species (Binder, 2010). Therefore we can hypothesize that there is a relationship between the amount of amino acids synthesized and aliphatic glucosinolates produced. Mutating this gene will therefore lead to reduction in the glucosinolates produced leading to a susceptible phenotype.

The ***at1g79460*** T-DNA mutant showed a lower reduction in biomass than the wild type Col-0 (Two way ANOVA, p=0.005) (Figure 3F). This suggests that the AT1G79460 gene improves *A. thaliana* resistance against *P. rapae*herbivory. This gene encodes for a protein with ent-kaurene synthase B activity which catalyses the second step in the cyclization of GGPP to ent-kaurene in the gibberellins biosynthetic pathway. Gibberellin (GA) is one of the most important hormones in plants, it regulates growth and influence various developmental processes such as flowering, sex expression, stem elongation, germination and seed development(Huang et al., 2012). The enzyme ent-kaurene plays an essential role in the three stages of GA biosynthesis in higher plants. GA plays a role in plant immunity signalling through the degradation of the nuclear family transcription factors known as the DELLA proteins. These proteins are thought to be involved in the modulating the balance between SA and JA signalling during plant immunity response. They suppress the SA and promote the JA and thus

boost the plant resistance to necrotrophs and susceptibility to biotrophs(Pieterse et al., 2012). A mutation in the AT1G79460 locus (ga2-1) results in reduced biosynthesis of GA (M Koornneef & Van der Veen, 1980) leading to an enhance accumulation of DELLA. The DELLA suppresses the JA signalling repressor JASMONATE-ZIM-DOMAIN(JAZ) by binding to it and hence reducing the JAZ and basic helix–loop–helix (bHLH) transcriptional factor (MYC2) interaction. This allows the MYC2 to stimulate a higher JA responsive genes(Pieterse et al., 2012).Studies have shown that *P. rapae* induces JA and ET and this suggest that these hormones play a role in defence against *P. rapae*and hence a resistant phenotype in this mutant.

The rest of the mutants did not show any significant interaction between genotype and treatment. This could be due to leaky mutants or genetic redundancy. P values from the two way ANOVA (Genotype × Treatment) for all mutants are summarized in Table 1.

3.2 Two choice bioassays.

For ***at2g2410*** T-DNA mutant,*P. rapae* larvae did not significantly prefer any of the genotype before infestation (Chi-square, p-value = 0.108). However, after 72 hours infestation, the larvae significantly preferred the wild type plants (Chi-square, p-value ≤0.001) (Fig 2). For ***at3g11480*** the *P. rapae* larvae did not show any preference.

Mutant ***at2g24210*** was less attractive than wild type after *P. rapae* infestation. AT2G24210 is also known as terpene synthase 10 (TPS10) and encodes a TERPENE SYNTHASE which is involved in the synthesis of sesquiterpenes and monoterpenes volatiles. These are herbivory induced volatiles (HIV) and can attract the natural enemies and research has showed that over expression of TPS10 in *A. thaliana* attract natural enemy of a lepidopteron larva, *Cotesiamarginiventris*(Schnee et al., 2006*)*. TPS10 gene is known to synthesis (-)-(3R)-linalool as its main product. Linalool is a monoterpene present in the floral scent (Ginglinger et al., 2013)and is also a HIV (Yang et al., 2013). The mutant lacked the linalool and thus it did not attract the larvae. This suggests that ***at2g242210*** can contribute to antixenosis effect in *Arabidopsis.*

4 CONCLUSION:

This study has identified four genes that when disruptedleads to either resistance or susceptibility of*Arabidopsis thaliana* in response to specialist insect *P. rapae*in comparison to the wild type. I recommend overexpression of these genes to see the resulting phenotype and further test the hypothesis that I proposed here. The knowledge gained could be transferred to the crop plants to boost their resistance against *P. rapae.*

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Table 1:P values for non-choice experiments for the genes tested. Two way ANOVA was carried out and the P-values for the interaction between Genotype × Treatment presented. Red color indicate p-values ≤ 0.05

Shoot FW		
GENE	**LINE**	**P. value**
AT1G09920	N658754	<.001
AT1G09920	N658754	0.050
AT1G10060	N676362	0.4090
AT1G10050	N654139	0.8720
AT1G10060	N670606	0.7024
AT1G10070	N537854	0.0027
AT1G10090	N653562	0.0380
AT1G10090	N653562	0.0070
AT2G43020	N660420	0.6790
AT2G43020	N669768	0.7160
AT2G43020	N682301	0.9310
AT1G79460	N677625	0.0050
AT1G79460	N677625	0.6210
AT1G79530	N653315	0.4900
AT1G79530	N684841	0.3310
AT2G24210	N662209	0.9010
AT2G31880	SOBR1	0.6520
AT2G42870	S022002	0.8240
AT3G11480	ATSBMT1	0.0760
AT3G24982	RFLP40	0.8610
AT3653240	RFLP45.2	0.4589
AT3G53240	RFLP45.1	0.5304
AT3G57260	N587824	0.1838
AT5G07690	N121027	0.6690
AT5G07690	H210512	0.0600
AT5G07700	N662521	0.9780

AT2G36590	N583340	0.9735
AT3G49120	N679662	0.0839
AT1G70820	N672942	0.8309
AT1G70820	N679092	0.4224
AT1G77850	ARF17	0.9100

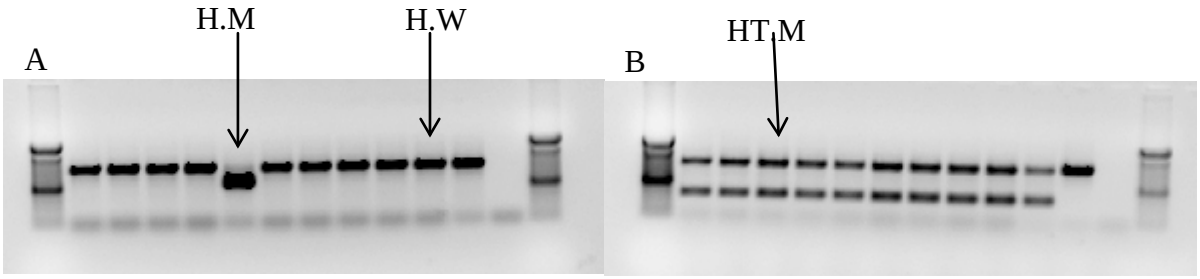


Figure 1: Band patterns on a gel photo. On gel A, H.W means homozygous wild type and H.M means homozygous mutant. On gel B, HTM means heterozygous mutant.

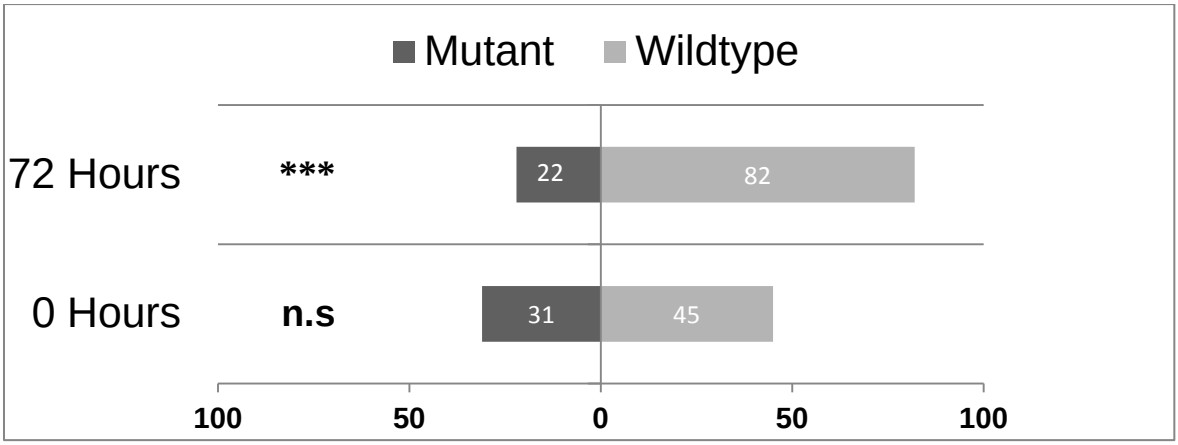
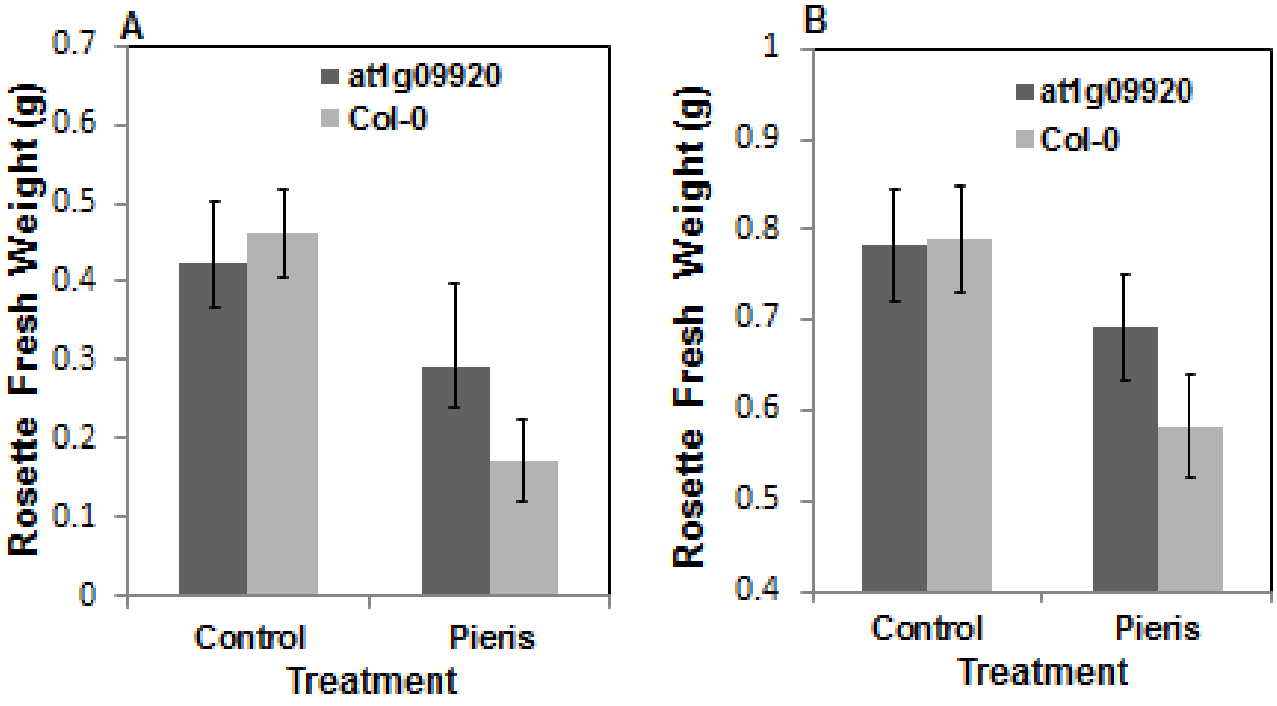


Figure 2:Preference of *P. rapae* in a two choice tests for AT2G2410. For each test, 13 pairs of genotypes were used. Bars represent the number of herbivores that preferred the phenotype. Numbers in bars represent numbers of larvae that made the corresponding choice. Data were analysed using Chi-squire test. Significant differences: ***, P ≤0.001 was found after 72 hours of infestation and n.s, no significant differences was found before the infestation with *P. rapae*. On each time point, a total of 130 larvae were tested.



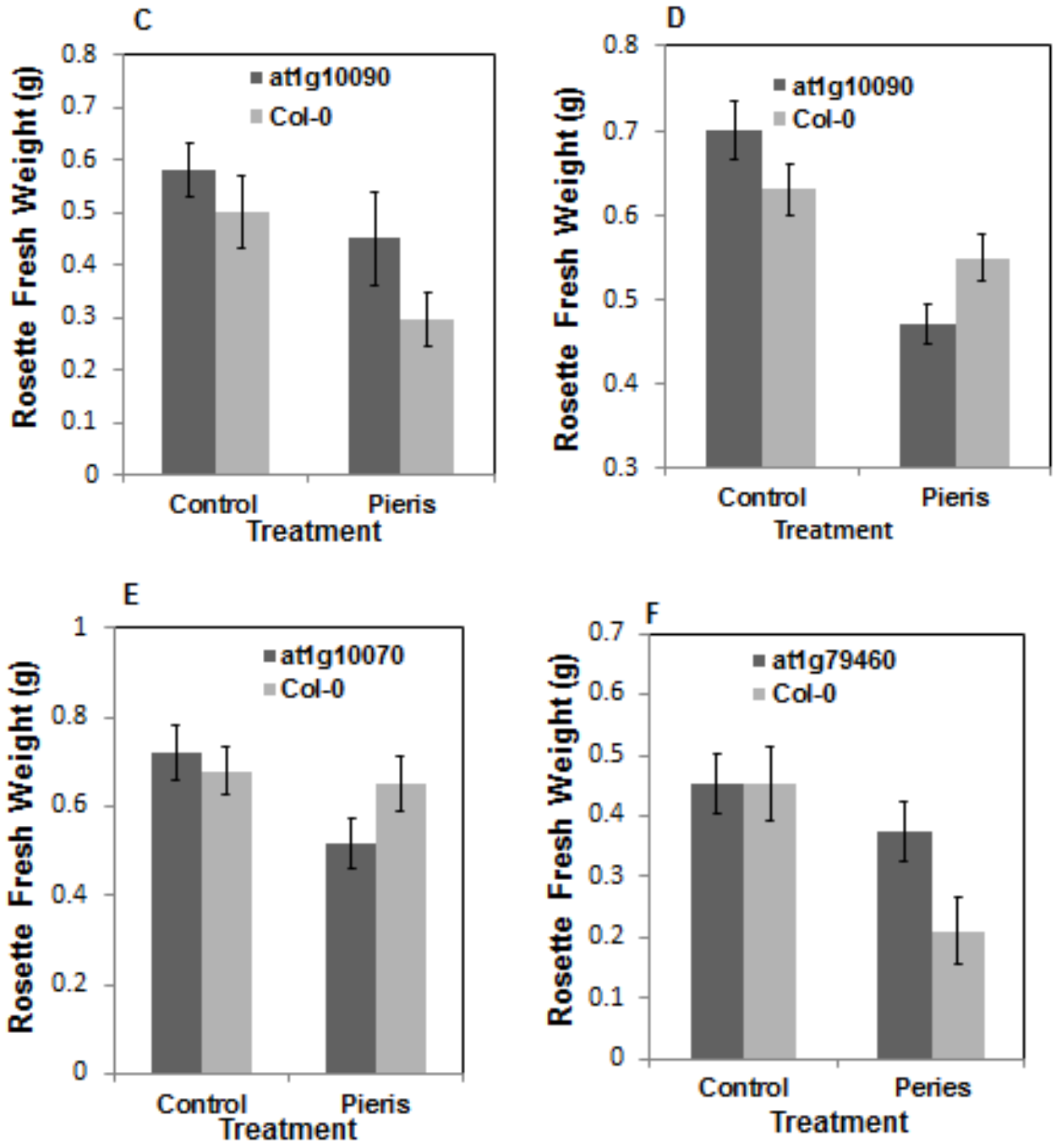


Figure 3: Mean ± SE Rosette fresh weight (g) of *A. thaliana* T-DNA mutant and Col-0 treated with or without *P. rapae* per test that showed significance difference.A and B shows two test of gene AT1G09920 with P. values of <.001 and 0.058 respectively. C and D shows gene AT1G10090 with P. values of 0.038 and 0.007 respectively. E shows gene AT1G10070 and F shows gene AT1G79460 with P. values of 0.00273 and 0.005 respectively.

6 SUPPLEMENTARY INFORMATION (SI):

SI1:Simple genomic DNA isolation (Jacqueline Busscher)

1. Put app. 8 glass balls into 1 ½ ml safe-lock eppendorf tube, add 1 small arabidopsis seedling leaf, put into N2 (l). Grind up leaf tissue in a shaker (3M ESPE Capmix) for 10 seconds.
2. Add 250 ul Shorty Buffer: 0.2M Tris/HCl pH9.0, 0.4M LiCl, 25mM EDTA, 1% SDS
3. Spin 10 minutes at high speed in micro-centrifuge
4. Transfer 175 ul of supernatant to a fresh eppendorf containing 175 ul isopropanol.
5. Mix by inversion and spin 10 minutes at top speed in micro-centrifuge
6. Pour off liquid and dry pellet by letting it sit upside down on a paper towel. You should not expect to see a pellet, but there is DNA, so don't worry.
7. Once the tube is dry add 100 ulTris buffer pH8 and re-suspend by shaking at room temp for 5 minutes.
8. After pellet is dissolved, spin 10 minutes and take the sup.(app 90 ul)
9. Use 2 ul of prep per PCR-reaction.

SI 2: A complete candidate gene list showing the gene,line and the primer set used in the genotyping.

Gene	Line	Primer LB	Primer RB
AT1G09920	N658754	TCTGGGATTATTTTGTTTACGTG	CAATGTACACGATGCAGATCG
AT1G10050	N654139	CAACAGCGAAGATCTGGAGAC	CTGGAACCTGTTTTGCTTCTG
AT1G10060	N676362	ATGCAGATGGTGAGGTTAACG	CAGCGACGAAGAGCCATATAC
AT1G10060	N670606	AGTGGTTGAGTTTTGTGGACG	CAGCGACGAAGAGCCATATAC
AT1G10070	N537854	AGATCTGTCTGATCAGGGCAG	TTTTACCCAACGTTTGTTTGC
AT1G10090	N653562	ATGCAGGGACAATCAGACAAC	CAAGATGCGTATAGTCGGAGG
AT1G10095	N523210	AACCTCGCCTATTGATTCCAC	GCGAAATCCCTAATTCTACCG
AT1G70820	N679092	AAAACAAAATCGCAATGCAAC	TACTCTGCCTCATATGCACCC
AT1G70820	N672942	AAAACAAAATCGCAATGCAAC	TACTCTGCCTCATATGCACCC
AT1G77850	ARF17	-	-
AT1G79460	N677625	TCAGGGCATTCATTCTCATTC	TGTTGGATTTAGGCGTCTTTG
AT1G79520	N657426	ACAAAATCAGCAGAGCGAGAG	GAGTCCAAAGTGGAAGCAATG
AT1G79530	N684841	AACTGTCCACGCAACTACAGG	CAAAGCTAACGATGCGCTATC
AT1G79530	N653315	TTTCCTTCCAAGACCTGTTCC	ATTCAAGCAGATCAAAGCACG
AT2G24210	N662209	-	-
AT2G31880	sobir1	-	-
AT2G42870	N522002	-	-
AT2G43020	N660420	GTACTAGCGGTGACAATTCCG	GAAATTTGCAGCTGCTTCATC
AT2G43020	N669768	TGTGGCTAGGGTTTTGATTTG	GATGAAGCAGCTGCAAATTTC
AT2G43020	N682301	CACTTTTGCAAGCTTGGTTTC	TCAATCCAGTTGAATAAGCGC
AT3G11480	ATSBMT1	-	-
AT3G24982	RFLP40	-	-
AT3G53240	RFLP45.2	-	-

AT3G53240	RFLP45.1	-	-
AT3G57260	N587824	TACAAGCAATGCAGAACATCG	TCTCGCTGTTTACAACGTTCC
AT5G07690	N121027	-	-
AT5G07690	H210512	-	-
AT5G07700	N662521	CCTTGATGTAGAGCTCGATCG	ATTGTCACGAGTGCATGACAC