ARGONAUTE4 (AGO4) IS INVOLVED IN ARABIDOPSIS RESISTANCE AGAINST GREEN PEACH APHIDS

A Thesis

by

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ABSTRACT

AGO4, one of the ten AGO proteins in *Arabidopsis*, is involved in gene silencing at transcriptional level. The general objective of this research is to understand whether AGO4 protein plays a defense role against *Myzus persicae*(the green peach aphid) infestation. The green peach aphid (GPA), as a generalist sap-sucking insect, is an economically important agricultural pest, which has broad host range among 40 different plant families including important crops like potato, sugar beet, and stone fruits. Understanding the mechanism of AGO4 in the aphid resistance will help us design effective pest control strategies. This study intended to identify potential AGO4-regulated genes in response to GPA infestation. qPCR analyses were performed on GPA fed on *ago4* and Col-0 plants. Genes exhibiting different expression patterns between *ago4* and Col-0 were selected to study their biological functions. Among 127 aphid response genes, 25 genes were selected and bioassays were performed on 38 selected T-DNA mutant lines. *ago4-1* mutant plants showed compromised resistance to GPA. Adult aphids raised on *ago4-1* mutants displayed higher fecundity than those raised on wild-type plants. At5g17990, the TRYPTOPHAN BIOSYNTHESIS 1 (*TRP1*) gene was induced in wild-type plants but decreased in *ago4-1* mutant plants after aphid infestation. Moreover, our bioassay experiment indicated that

the mutant plants of *TRP1* were preferred by GPA, suggesting *TRP1* gene could be an aphid resistant gene, possibly regulated by AGO4.

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CHAPTER I

INTRODUCTION AND

LITERATURE REVIEW

I.1 Introduction

Aphids are one of the most destructive pests to plant crops both as direct pests and as pathogen vectors. Around 100 species of significant economic importance have successfully adapted to the agricultural environment (van Emden & Harrington 2007) For example, *Acyrthosiphon pisum*(pea aphid), is globally distributed throughout all temperate regions of the world and feeding on many species among 13 plant families including over 200 species of Fabaceae.It is particularly important on peas, beans, alfalfa and clover and is a vector of more than 30 virus diseases, such as *Peaenation mosaic virus* (PEMV) and*Bean leaf roll virus* (BLRV)(Caillaud 1999).*Aphis craccivora* (cowpea aphid), attacks 50 crops in 19 different plant families, including Fabaceae and many other plant families. In addition, it transmits 30 plant virus diseases, including non-persistent viruses of beans, cardamom, groundnuts, peas, beets and the persistent *Subterranean clover stunt virus*, *Peanut mottlevirus* (van Emden & Harrington 2007). *Aphis gossypii*(cotton aphid) can occur in large groups, on over 100 species of crop plants, including cotton, cucurbits, coffee, potato, peppers. Cotton aphid can vector more than 50 plant viruses, such as

anthocyanosis virus, *Lily symptomless virus*,PEMV, and lily rosette disease(Thomas et al. 2001).*Macrosiphum euphorbiae* (potato aphid), feeds on more than 200 plant species in 20 different plant families, and transmit more than 40 non-persistent and five persistent viruses, such as BYNV, PEMV, BLRV, *Sweet potato leaf-specking virus*, ZYMV (Margaritopoulos et al. 2005).*Schizaphis graminum* (greenbug) has a significant economic effect in the winter wheat areas of North America. They feed on the leaves of grasses and cereals and cause yellowing and other phytotoxic effects. Their main host is almost exclusively to Poaceae and they transmit several important viruses, including BYDV, MRLV, SCMV and MDMV (van Emden & Harrington 2007). To control aphid damage to crops and find an environmental friendly method for pest management, it is necessary to study the mechanism of plant defense against aphids.

I.2 Plant-Aphid Interactions

Being sessile, plants have acquired the ability to adapt and respond to a variety of stresses, including those from other organisms (biotic) and the environment (abiotic). Abiotic stresses include exposure to extreme temperatures, drought and flooding, etc. Biotic stresses include attacks by bacteria fungi, insects and nematodes. Insect herbivory causes 10-20% loss of crop yield annually (Boyko et al. 2006). Based on the feeding mode, insect pests can be categorized into two groups: (i) The chewing insects, which use their shear-

like mouth parts to cut and chew the foliar tissue, leading to extensive plant wounding and tissue loss (Kandoth et al. 2007),and (ii) the piercing-sucking insects, which utilize their modified mouthparts, slender stylets, to feed from sieve elements and drink large volumes of phloem sap(Kaloshian & Walling 2005). Aphids (Hemiptera: Aphididae) constitute the major group of piercing-sucking insects that tap into sieve elements following an intercellular route. During feeding, aphids produce two types of saliva, gelling saliva and watery saliva. The gelling saliva forms a tight sheath around the stylet during penetration, which can minimize plant reactions for the isolation of stylet from the host cells (Tjallingii 2006). The watery saliva contains a variety of hydrolytic enzymes like pectinases, cellulases and other enzymes like polyphenoloxidases, glucose oxidase and peroxidases. These enzymes help aphids repress plant defense responses (Harmel et al. 2008) and prevent sieve element occlusion. The mainly intercellular route taken by stylets, combined with aphid salivary components, minimizes physical damage to plant tissue and provides the basis of the sophisticated aphid-plant interaction.

I.2.1 Effects of Aphid Infestation on Host Plants

Aphid infestation leads to reprograming of plant metabolism, including carbohydrate metabolism, premature senescence and activation of plant hormones (Louis et al. 2012). For example, aphid infestation alters source-sink patterns in the infested plant, leading to

an increasing flow of nutrients to the infested organs instead to the natural sink tissues. The concentrations of sucrose and starch increase in infested leaves of *Arabidopsis* after GPAinfestation. Also, aphid settlement on growing zones of the stem results in a reduction of C and N fluxes at the apical zoneof the stem (Girousse et al. 2005). GPA infestation also results in the activation of cell deathin *Arabidopsis* (Pegadaraju et al. 2005). It has been reported that aphid feeding on host plants activates salicylic acid (SA) and jasmonic acid (JA) biosynthesis pathways (Moran & Thompson 2001). The expression of lipoxygenase (LOX), one of the enzymes involved in the biosynthesis of JA, was induced by*Myzus nicotianae* (tobacco aphid) in tobacco plants. SA also increased under aphids attack, e.g. expression of SA biosynthesis and responsive genes was observed in bluegreen aphid infested *M. truncatula* plants (Gao et al. 2007).In addition, SA responsive genes like *PR-1, BGL2* and *PDF1.2* in *Arabidopsis*were induced by GPA (Moran & Thompson 2001).

I.2.2 Plant Defense Responses against Aphids

To counter aphid's infestation, plants have evolved multiple defense mechanisms, which can be broadly grouped three categories: antibiosis, antixenosis and tolerance (Smith 2005). Antibiosis refers that plant resistance adversely impactsaphid development, reproduction and/or survival (Smith 2005). Antixenosis is a resistant mechanism rendering insects unable to select the plant as a host due to both physical and chemical cues on the

plant (Smith 2005). Tolerance is the ability of plant to withstand and recover from damage caused by aphids. In addition, plants can also produce volatiles during aphids infestation and attract predatory insects to control aphid infestation, for example, methyl salicylate (MeSA) is a soybean aphid-induced plant volatile, which attracts the predatory beetle, *Coccinella septempunctata*, to the infested plants to control aphid population (Zhu & Park 2005).

I.2.3 Gene-For-Gene Interaction in Defense against Aphids

Likeresistant (R) genes involved in plant-pathogen interactions, a similar gene-for-geneinteraction has been shown intomato, the *Mi-1*gene belongs to the *Mi-1*nucleotide binding site (NBS)-leucine rich repeat (LRR), it has been shown that tomato plants carrying the *Mi-1* gene are more resistant against the potato aphids compared to the plants lacking this gene (Martinez de Ilarduya et al. 2003). Also, another NBS-LRR member, AKR (*Acyrthosiphon kondoi resistance*) was required for *Medicago truncatula* resistant against the bluegreen aphid (Klingler et al. 2005).

I.2.4 Reactive Oxygen Species (ROS) in Defense against Aphids

Generation of H_2O_2 during aphid infestation could enhance plant defenses due to the production of antioxidants in plant cells. Also, genes encoding for ROS accumulation were

up-regulated in the greenbug infested leaves of *Sorghum bicolor* (sorghum) plant. The H_2O_2 burst and the increasing amount of peroxidases may strengthen cell wall and increase plant resistance against aphid (Moloi & van der Westhuizen 2006).

I.2.5 Involvement of JA in Defense against Aphids

It has been shown that *Macrosiphum euphorbiae* feeding on tomato plants and *M. persicae* feeding on *Arabidopsis* elevated the mRNA expression of LOX, one of the enzymes involved in JA biosynthesis (Moran & Thompson 2001). Also, the feeding activity of Russian wheat aphid and greenbug on wheat and sorghum plants respectively lead to a higher expression of *LOX* genes (Boyko et al. 2006). The application of MeJA, the methyl ester of JA, on plants could reduce aphid fecundity, as shown in MeJA treated of alfalfa and tomato (Gao et al. 2007). Also, the MeJA application could reduce aphid infestation on sorghum (Zhu-Salzman et al. 2004). Moreover, JA hyper-accumulating *cev1* mutants of *Arabidopsis* enhanced resistance to GPA (Ellis, Ellis, et al. 2002). Thus, both JA and MeJA are important for plant defense against aphids.

I.2.6 Involvement of SA in Plant Defense against Aphids

SA levels were up-regulated in barley during the attack of *S. graminum* (Chaman et al. 2003). Also, GPA infestation led to the up-regulation of SA responsive genes like *PR-1*,

BGL2 and *PDF1.2* in *Arabidopsis* (Moran & Thompson 2001), indicating the involvement of SA in plant defense against aphids. However, the study of GPA feeding on the SA biosynthesis mutant *sid2* (salicylic acid-induction deficient2) and on SA insensitive mutant *npr1* (non-expressor of PR-1) indicated that aphid populations were comparable to that on wild-type plants, which suggested that SA is not important for *Arabidopsis*defense against GPA. The role of SA in plant-aphid interaction may differ depending on the plants and insects involved. For example,*fad7* mutant of tomato accumulating higher level of SA results in enhanced resistance to potato aphid (*Macrosiphum euphorbiae*) (Avila et al. 2012). Since SA signaling is able to attenuate the activation of JA signaling, it was proposed that aphids could mislead host defenses by inappropriately activating SA signaling and depressing the JA signaling, which facilitated insect infestation (Avila et al. 2012).

I.2.7 Involvement of Ethylene (ET) in Plant Defense against Aphids

Both ethylene biosynthesis and responsive genes were up-regulated in *M. truncatula* after bluegreen aphid infestation (Gao et al. 2007). In addition, the feeding activity of *Schizaphis* and *Rhopalophum padi* on barley plants led to an increasing level of ET production (Argandoña et al. 2001). Also, GPA feeding could result in the up-regulation of genes involved in ET biosynthesis in *Arabidopsis* (Moran et al. 2002).

I.3 Model Species

Arabidopsis thaliana has been used as a model plant to study plant growth, development and response to stresses (Koornneef & Meinke 2010). The small size, short generation time, the completely sequenced genome, ease for transformation and available mutants make *Arabidopsis* ideal for molecular-genetic studies(Koornneef & Meinke 2010).

Myzus persicae, a generalist sap-sucking insect, is an economically important agricultural pest, which has broad host range among 40 different plant families including important crops like potato and sugar beet, stone fruits (Blackman & Eastop 1984). GPA can severely reduce crop yields by direct consumption through plant phloem sap and by vectoring more than 100 viruses. Moreover, GPA developed resistance to a large number of insecticides (Georghiou & Lagunes-Tejeda 1991). Thus GPA has been categorized amongst the top three agricultural pests in the USA (Koch & Waterhouse 2000). The interaction between *Arabidopsis* and GPA has been used to characterize plant response to phloem-feeding aphids and to find plant genes and mechanisms involved in plant defense to aphids (Louis et al. 2012). In our study, we employed the *Arabidopsis*-GPA system to study the nature of plant-aphid interaction.

I.4 Defense Mechanisms of *Arabidopsis* Resistance against GPA

During the past few decades, some mechanisms involved in *Arabidopsis* resistance against GPA have been attained. For instance, the phloem sap of *Arabidopsis* contains an antibiotic factor that is detrimental to GPA. Glucosinolates, the defensive compounds contained in Brassicaceae family plants including *Arabidopsis*, involved in plant resistance against GPA. The *Arabidopsisatr1D* mutant that accumulates higher amounts of indole-glucosinolates than wild-type plant showed an elevated resistance to the GPA (Kim et al. 2008). Double mutant *cyp79B2 cyp79B3* that does not accumulate indole-glucosinolates were susceptible to the GPA (Pfalz et al. 2009). The constitutive expression of PP2-AI, a plant lectin in phloem, adversely impacted the ability of GPA feeding from the sieve element (C. Zhang et al. 2011). Non-protein amino acidswere also involved in plant resistance against aphids. For example, GPA fecundity was significantly reduced when aphids were fed on artificial diet containing N^{δ}-acetylornithine, a novel class of non-protein amino acid. Also, *Arabidopsis* lipids were involved in plant defense against GPA. Mutation of α -dioxygenases (α -DOXs), failing to yield oxylipins (oxidized lipids) resulted in increased susceptibility to aphids and GPA population size was larger on the *Arabidopsisα -dox1* mutant (Avila et al. 2012). Although, GPA infestation activates SA signaling in *Arabidopsis*, the SA signaling is not critical for controlling GPA. For example, GPA did not exhibit increased colonization on the *ics1* and *eds5* mutants, ICS1 and EDS5

are both involved in biosynthesis of SA. Also, mutation in the NPR1, a key SA signaling regulator, did not result in improved performance of GPA on *npr1* mutant than the wild-type plant. Although, aphids may have evolved to activate SA signaling and repress JA signaling, the mutant *cev-1*, which contains higher levels of JA than wild-type, was more resistant to GPA. In addition, the exogenously applied MeJA also promote resistance to GPA in *Arabidopsis* (Ellis, Karafyllidis, et al. 2002). The *ArabidopsisPAD4* gene, which encodes a nucleocytoplasmic protein, is required for plant defense against GPA both in antibiosis and antixenosis. For instance, when given a choice between the wild-type and *pad4* mutant, GPA preferred to settle on the mutant plants and when given a choice between wild-type and *PAD4* over-expressing plant, GPA preferred to feed on wild-type plant (Pegadaraju et al. 2007). Also, the GPA population was significantly larger on the *pad4* mutant and lower on PAD4 over-expressing plants than wild-type plants (Pegadaraju et al. 2007). Recently,sRNAs was found to be involved in *Arabidopsis* defense against GPA. Genetic studies found that GPA fecundity was significantly lower on plants with mutations in the genes involved in miRNA generation, such as *DCL1*, *HEN1* and *AGO1* (Kettles et al. 2013).

I.5 ARGONAUTE4 (AGO4): Involvement in Plant-Aphid Interaction

I.5.1 AGO4 is involved in Small RNAs (sRNA) Processing and DNA Methylation

Argonaute proteins are components of the RNA-induced silencing complex (RISC), in multiple organisms (Law & Jacobsen 2010). In *Arabidopsis*, RNA-mediated silencing is triggered by small RNA (sRNAs), Based on their origin and mode of processing, sRNA can be classified into subgroups. siRNAs are derived from segments of long perfectly complementary dsRNA, and microRNAs (miRNAs) belong to a class of largely 21-nt sRNAs, which are processed from imperfectly complementary stem-loop precursors (Vázquez et al. 2010). For miRNAs biogenesis, a MIR gene is transcribed into pri-miRNA and then processed into pre-miRNA by DCL protein, which is normally DCL1 in *Arabidopsis*. miRNAs duplex is produced with the help of RNA pol II, Dicer-like 1(DCL-1) and Hyponastic Leaves 1 (HYL1). The miRNA-miRNA* duplex is methylated by HUA ENHANCER1 (HEN1). One strand of miRNA duplex is loaded into RISC containing AGO1 protein. miRNA is transferred from nucleus to cytoplasm by HST (HASTY) protein. For siRNA biogenesis, there are two types of siRNA precursors: non-coding regions for Ta-siRNAs and heterochromatic siRNAs. dsRNAs are transcribed from single-stranded RNA (ssRNA) by RNA-Dependent RNA Polymerases (RDRs). dsRNAs are sliced by DCLs to form siRNAs duplex, which is then methylated by HEN1 and unwound by helicase. The binding of siRNA to RISC directs the degradation of complementary target

mRNA (Xuemei Chen 2009). AGO4 is involved in gene silencing at transcriptional level in a process called RNA-directed DNA methylation (RdDM) (Zilberman et al. 2003; Zilberman et al. 2004). In *Arabidopsis*, siRNAs are produced by RNAse III enzyme DICER-LIKE 3 (DCL3) from its corresponding dsRNAs, siRNAs is then loaded to AGO4 to form an AGO4-siRNA complex, the AGO4 protein is thought to recruit the protein DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), which catalyzed *de novo* DNA methylation at symmetric CG or CHG and asymmetric CHH sites (Havecker et al. 2012).

A few members of AGO family are reported to be involved in plant response to pathogens. In PTI, PAMP (flg22)-induced callose deposition, gene expression, and seedling growth inhibition are AGO1-dependent. A lot of AGO1-bound sRNAs are up- or down-regulated by flg22, such as miR160a. Overexpression transgenic plant of miR160a shows higher callose deposition under flg22 treatment (X. Zhang et al. 2011). Recently, people found that *Arabidopsis* AGO2 functions in innate immunity against bacterial pathogens. AGO2 is highly induced by *Pseudomonas syringa*. miR393b*(one of abundant species among AGO2-bound *sRNA*) represses expression of a Golgi-localized SNARE gene, *MEMB12*. Loss-of-function mutant of *MEMB12* has increased amount of PR1 (Zhang, Zhao et al. 2011). AGO4 was involved in plant immunity system, two loss-of-function alleles *ago4-*

1 and *ago4-2*, show more susceptible to virulent *Pseudomonas syringae* and also to the avirulent *P.s.t.* DC3000 carrying the effector *avrRpm1* gene. This susceptibility is SA-independent (Agorio & Vera 2007).

I.5.2 DNA Methylation in Plant Defense

DNA methylation refers to the addition of a methyl group to the cytosine bases of genomic DNA to form 5-methylcytosine (He et al. 2011). In *Arabidopsis*, genome is methylated at CG, CHG, and CHH (where H=A, T, or C) sequence contexts (Law and Jacobsen 2010). It is well known that DNA methylation, as a stable and heritable epigenetic mark, plays an important role in transposable element silencing and gene imprinting (He et al. 2011). In many eukaryotes, regulation of gene expression by DNA methylation is important for organism development, and at molecular level, DNA methylation is coupled with nucleosome positioning, specific histone modifications and transcriptional activity. For instance, in *Arabidopsis*, the disruption of the chromatin remodeling enzymes KYP, SUVH5 and SUVH6 result in decreased DNA methylation and transcriptional reactivation of heterochromatic transposons. Recently, modification of DNA methylation profiles in response to environmental stresses, which leads to the transcriptional activation of defense-related genes, has been proposed to be one of plant immunity mechanisms. Mutants*met1-3* and*drm1-2 drm2-2 cmt3-11*, almost eliminated in CG methylation and *de*

novo methylation, respectively, leading to genome-wide hypomethylation and development defects (Stroud et al. 2013). The *met1* and the *ddc* mutants were more resistant to bacterial *DC3000*compared to wild-type.An RNA-seq experiment found that many pathogen-responsive genes were constitutively expressed in *met1* and *ddc*, e.g. PHYTOALEXIN DEFICIENT4 (*PAD4*)were up-regulated in *met1* mutant by 2.42 foldsand in *ddc* 4.03 folds. Moreover, by comparing the methylomes of untreated and *Pst*-treated *Arabidopsis* plants by whole genome bisulfite sequencing, many cytosines at CG and CHH sites became differentially methylated regions (DMRs), it was also found that the DMRs are associated with genes involved in defense response, and hypomethylated DMRs is accompanied by up-regulation of corresponding genes, particularly those defense response genes. It was also reported that some plant NOD-like receptors (NLRs), which encode key immune receptors, were regulated by DNA methylation. For example, one of the NLR family genes, the *Resistance Methylated Gene 1* (*RMG1*, *At4g11170*), expressed higher in basal *met1* mutant compared to wild-type plant, however, the DNA methylation levels at the promoter region of *RMG1* is significantly lower than wild-type plant. Thus, DNA methylation could be partially involved in regulation of pathogen defense-related genes. In addition, many proteins involved in regulation ofplant DNA methylation have been implicated in plant innate immunity. For example, two loss-of-function alleles of AGO4 in *Arabidopsis*, *ago4-1* and *ago4-2*, displayed compromised

resistance to virulent *Pseudomonas syringae* and to the avirulent *Pst* DC3000 carrying the effector *avrRpm1* gene(Agorio & Vera 2007).

I.5.3 sRNAs in Plant Defense

sRNAs are short, noncoding RNA molecules that regulate silencing of genes through transcriptional gene silencing or post-transcriptional gene silencing. sRNAs regulate many cellular processes, including development, stress responses and metabolism. In addition, accumulating evidence showed that sRNAs and core pathway components are involved in plant immunity against various pathogens, such as bacteria, fungi, oomycetes and viruses. Many components associated in sRNA pathways are involved in defense responses to pathogens (Seo et al. 2013).

Compromised response of *ago1* in PTI is due to the positive regulation of PTI by miR393. For instance, miR393 targets mRNAs encoding the auxin receptor, transport inhibitor response 1 (TIR1), which results in negatively regulation of auxin signaling and shifting energy from growth to basal defense. Repression of auxin signaling restricts *P. syringae* growth (Navarro et al. 2006). In *ago1* mutant, failure loading on AGO1 of miR393 blocked TIR1 regulated Auxin signaling and resulted in compromised response of *ago1* in PTI (Ruiz-Ferrer & Voinnet 2009). Also, it has been found that AGO2 plays an important role

in antibacterial resistance. *ago2* mutant showed enhanced susceptibility to virulent and avirulent *P. syringae pv. tomato* strains. AGO2 functions in antibacterial resistance by binding miRNA393b* to regulate antimicrobial PR proteins via a Golgi-localized SNARE gene, *MEMB12*(X. Zhang et al. 2011). Ellendorff et al. (2009) found that AGO7 positively regulates fungal resistance and *ago7* mutant displayed enhanced susceptibility to fungal *Verticillium spp*. AGO7 functions with miR390, which triggers expression of TAS3 ta-siRNAs regulating expression of auxin-signaling components. In addition, AGO4 is required for bacterial resistance in *Arabidopsis*, *ago4* mutants compromised in the resistance to virulent and avirulent *P. syringae.* Beside the function in pathogen resistance, AGO proteins are also involved in antiviral defense. The expression level of AGO1 is induced when plants are infected with viruses (Csorba et al. 2009). Mutants of *ago1* and *ago7* were compromised in the defense against viruses. It was also reported that AGO2 functions in defense against RNA viruses, including TCV, *Potato virus X*, CMV, and *tomato bushy stunt virus*(Harvey et al. 2011). In addition the resistant against bacteria, fungi and nematodes, sRNA pathways were also involved in resistance to insects. In tobacco, the mutant of RNA-directed RNA polymerases (RdRs) was highly susceptible to leaf-chewing solanaceous specialist *Manduca sexta* (Pandey et al. 2008). miRNA mutant plants, *ago1* and *dcl1* displayed a higher resistance against GPA, due to a higher accumulation of camalexin in miRNA mutants (Kettles et al. 2013).

Studies on *Arabidopsis*-GPA interaction will help us to understand mechanisms involved in plant resistance against GPA and to discover new targets for biological control. The objective of this research is to determine if AGO4 protein in *Arabidopsis*affects defense against GPA and identify target genes potentially regulated by AGO4 and responsive to GPA, qPCR was performed on *ago4* and Col-0 plants in the presence and absence of GPA. Also, to study the biological function of potential target genes, aphid bioassays were performed on selected T-DNA mutant lines.

CHAPTER II

MATERIAL AND METHODS

II.1 Materials

Arabidopsis thaliana ecotype Col-0 and *ago4-1*, Ler and *clk-st*were used. Landsberg erecta (Ler) is the background line of *ago4-1* and *clk-st*. SUPERMAN (SUP) is a flower specific gene regulating the boundary of the stamen and carpel whorls in *Arabidopsis*. *AGO4* is responsible for *SUP* gene silencing *and clark kent (*clk*)* is the epigenetic allele of *SUP* gene in *Arabidopsis* containing hypermethylated cytosines at *SUP* region. The phenotype of *clk-st* is similar to mutation of *SUP* gene, with increased numbers of stamens and carpels(Jacobsen & Meyerowitz 1997). AGO4 is identified by screening for suppressors of the *clk-st* allele, SUP gene is induced in *ago4-1* mutant (Law & Jacobsen 2010). In our study, both Ler and *clk-st* are applied as control plants with *ago4-1* mutant. Seeds were soaked with water and cold treated at 4 °C and then grown at 23°C/19°C (day/night) under a 12 hours light /12 hours dark cycle.

Phloem sap-feeding GPA were cultured on cabbage (*Brassica oleracea*) and maintained in an environmental chamber at 21ºC, 65% RH, and 12 h light /12 hdark cycle.

II.2 Methods

II.2.1 Gene Selection Based on Database

127 Genes responsive to aphid infestation were selected based on several published microarray data (Moran & Thompson 2001; De Vos et al. 2005; Kuśnierczyk et al. 2007; De Vos & Jander 2009) with a two folds cut-off. Then DNA methylation sites were identified according on published database (Stroud et al. 2013). 95 genes were selected based on the existing of DNA methylation sites from 127 GPA responsive genes.

II.2.2 Genotyping of Mutant Plants

Mutant plants corresponding to selected genes were ordered from the *Arabidopsis* Biological Resource Center (ABRC).For genotyping, leaves of four-week old plants were used for DNA extraction. The method of DNA extraction from Edwards et al (Edwards et al. 1991) was used. Briefly, 200 ul extraction buffers (200mM Tris (pH 7.5), 250 mM NaCl, 25 m EDTA and 0.5% SDS) were used to grind plant leaves, after 5 min 14,000 rpm centrifuge, the supernatant were precipitated in the isopropanol solution for 30 min in -20°C. Then after precipitation, solution were centrifuged again, pellet was washed with 70% ethanol for two times and dissolved with 40 ul autoclaved water, which is ready for genotyping. Genotypes were confirmed by a combination group of gene specific primer (LP and RP) and T-DNA primer (LB, ATTTTGCCGATTTCGGAAC) (Table S2).

II.2.3 Sample Preparation for qPCR Analysis

For GPA treatment, three-week old *Arabidopsis* plants (mutant and Col-0) were infested with GPA by transferring 40 apterous adults to each plant (De Vos et al. 2005). GPA is allowed to feed for 12h, 24h and 48h on plants (Pegadaraju et al. 2005). The control was mutant plants and Col-0 plants without GPA infestation. Whole rosettes were harvested at different time points with liquid nitrogen. To remove aphids, the infested plants were flushed with deionized water and control plants were treated the same. At least 6 plants were needed for each treatment; samples from each treatment were combined and ground as a pool. Three biological replicates were performed. Whole rosette was harvested with scissor, and plants were flushed with deionized water and the control group was treated the same. Harvested tissue was wrapped into aluminum paper and stored in -80 °C, which is prepared for qPCR analysis.

For qPCR, total RNA was extracted from the harvested plant tissue using a Trizol-based method (Invitrogen, Carlsbad, CA) and then was reverse transcribed with Superscript™ II reverse transcriptase (Invitrogen) by using random hexamer primers. Primers for amplifying selected genes were designed using Primer Express (Applied Biosystems) or published information. No-template controls using untranscribed RNA confirmed that no interfering products derived from genomic DNA were present. PCR amplification of 18S

rRNA was performed for normalization between treated and control samples. Amplification specificity was determined by dissociation curve analysis. 2^ (ΔΔCT) was applied to calculate mean induction fold.

II.2.4 Plant Damage Assays

For plant damage assays, at least 8 plants from each genotype were used. Seeds were cold treated under 4°C for at least two days, then which were saw into soil for germination, during this period, plants were covered with plastic coversto keep the humidity. Two weeks later, seedlings were transferred into individual pots, which were kept in growth chamber, which has the growing condition with 23°C/19°C under a 12 h light /12 h dark cycle. Five weeks old plants were infested with adult aphids, by considering the plant sizes of each genotype, 40 adult aphids were used. The phenotype of plants was observed each day and the symptoms including chlorosis, leaf rolling and plant decay were expected to show. Images were taken of representative leaves when the plants show the expected phenotype and control plants were recorded also. All experiments were repeated for three times.

II.2.5 Measurement of Plant Chlorophyll Contents

To measure the leaves chlorophyll contents, four weeks old *clk-st* and *ago4-1* plants were

inoculated with 40 adult aphids respectively. 10-days after infestation, aphid-induced chlorophyll loss was measured and the chlorophyll was extracted from entire rosette leaves of six control and aphid-infested plants of each genotype. The entire rosette leaves were ground with liquid nitrogen in a mortar with a pestle. Then chlorophyll was extracted with the extraction buffer, which consists of 85:15 (v/v) mix of acetone:Tris-HCl (1 M; pH 8.0 in water). The absorbance of the extracted solution was measured at 664 and 647 nm and extraction buffer was control.

II.2.6 GPA Bioassay Test

For no-choice test, at least 10 replicates of three weeks old plants were infested with aphid nymphs. Seeds were cold treated under 4°C for at least two days, then which were saw into soil for germination, during this period, plants were covered with plastic covering for keeping the humidity. Around two weeks later, seedlings would be transferred into individual pots, which were kept in growth chamber, which has the growing condition with 23°C/19°C under a 12 h light /12 h dark cycle. Six synchronized and second-instar nymphs were transferred to three-week old *Arabidopsis*, when most nymphs had reached adult stage and started to reproduce, the numbers of these new nymphs were counted on day 7. The newly produced nymphs were removed and the adults remained on the plant. On day 9, a second nymph count was carried out, together with a count of the surviving

adults. Experiments were terminated on day 9. Infested plants were kept in bioassay chamber with the condition of 23°C/19°C under a 12 hours light /12 hours dark cycle. We expect to find the transgenic lines, on which GPA has less progenies compared to wild-type.

For Choice Test, seeds were treated the same with the plants for non-choice test. Plants were growing under the same condition of non-choice test plants. At least 8 pairs of plants were used for choice test. Two genotype plants were linked with a piece of paper, 35 adults were released at an equal distance between two plants of different genotypes at three-week old stage. The number of adult aphids settled on each plant was counted at 6, 12 and 24 hours after releasing. This experiment was repeated for three times. We expect to find the RNAi lines that GPA does not prefer to settle and feed on compared to wild-type.

To determine GPA performance on Ler, *clk-st* and *ago4-1*plants,GPA nymph developmental times, nymph survival rate, adult fecundity and longevity of aphids feeding on different genotype plants were recorded. For aphid life table assay, at least 20 plants from each genotype were used. Seeds were cold treated under 4°C for at least two days, then which were transferred into soil for germination, then plant was transferred into individual pots, 2-3 weeks old plants were used for aphid development. For infestation,

adult aphids were transferred to *Arabidopsis* for laying young nymph and then were removed. In order to record the development time of each instar of aphids, exuvia of instar was checked every 6 hours until the instar reached next developmental stage and the developmental time of each instar was recorded. In addition, overall survival rate of nymph from new hatch to adult, mean progeny of single adult aphids hatch daily and mean longevity of adult aphids were recorded.

CHAPTER III

RESULTS

III.1 *Ago4-1* Mutant Plants are Susceptible to Aphid Infestation

To determine the biological function of AGO4 in GPA resistance, the mutant *ago4-1* was used for bioassay analysis. For plant damage test, results displayed that compared to *ago4-1* mutant aphid infestation didn't cause significant damage to the *clk-st* plant after 10 days infestation. However, *ago4-1* mutant plants displayed the disease symptoms more rapidly than the *clk-st* plants, such as chlorosis, leaf rolling and plant decay (Figure 1A). After 7 days infestation, *ago4-1* mutants started to show the damage phenotype. After 10 days infestation, *ago4-1* mutants displayed more severe damage phenotype than the *clk-st* plants. Plant damage was also recorded and scored every two days (Figure 1B). In addition, the chlorophyll loss caused by aphid's infestation in *clk-st* was less than 5% after 10d feeding and the chlorophyll contents were around 60% left in *ago4-1* mutant (Figure 1C) (Lei JX unpublished data).

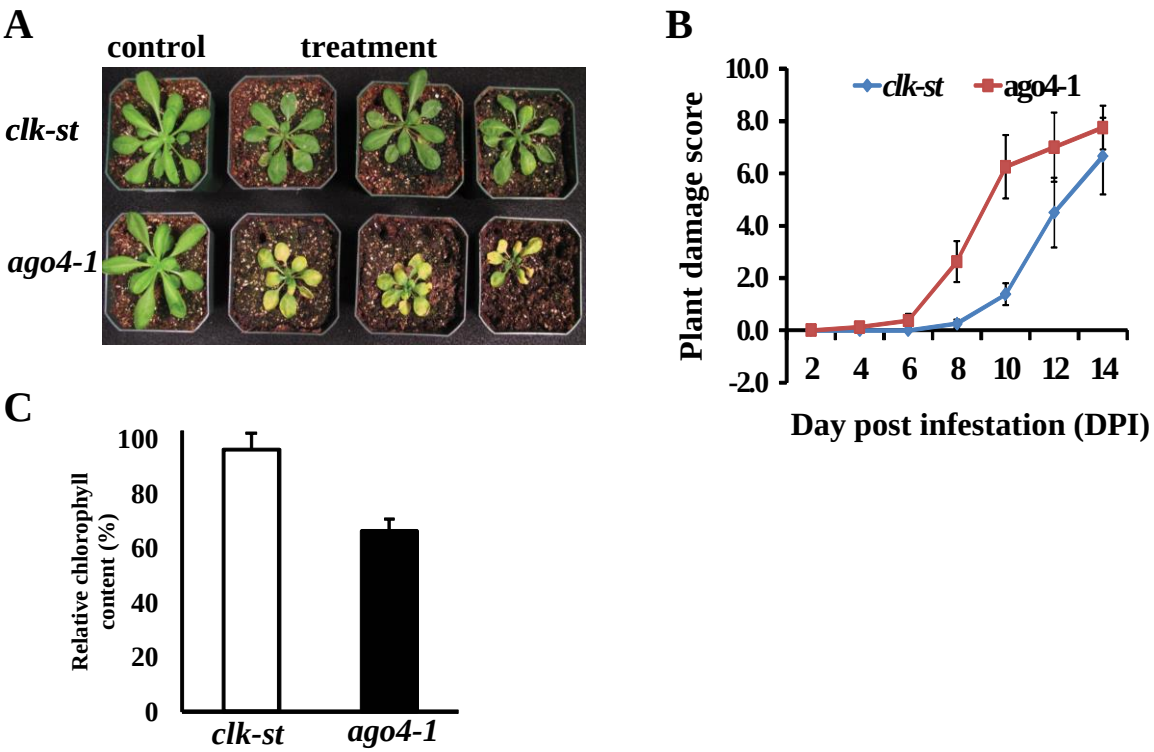

Figure 1. *Ago4-1* Mutant Plants Phenotype after Aphid Infestation.

A. Plant phenotype of *clk-st* and *ago4-1* after 10 days infestation with adult GPA
B. Plant damage score indicates an enhanced susceptibility of ago-1
C. Aphid-induced chlorophyll loss in *clk-st* and *ago4-1* mutant after 10 days infestation

III.2 Aphids' Performance on *Ago4-1*, *Clk-st* and Wild-type Plants

In order to test the biological function of AGO4 in GPA resistance, we recorded aphids performance feeding on *ago4-1*, *clk-st* and Ler plants, which includes nymph developmental times, nymph survival rate, adult fecundity and longevity of aphids. The developmental time from the fourth instar into adult on *ago4-1* was significant less than those aphids feeding on Ler and *clk-st* plants (Table 1). However, there were no significant

differences of the developmental time of aphids feeding on Ler, *clk-st* and *ago4-1*. In addition, aphids feeding on *ago4-1* mutants displayed a higher fecundity than those aphids feeding on *clk-st* plants, though no significant difference was detected between Ler and *ago4-1* mutant plants. In addition, aphids displayed similar performance on Ler, *clk-st* and *ago4-1* in respect of nymph survival rate and adult longevity. Thus, mutation of AGO4 affects aphid's performance and fecundity, but not the survival rate and longevity of aphids.

III.3 Gene Selection Based on Gene Expression and DNA Methylation Sites

In order to determine AGO4-dependent genes related to aphid defense, 127 aphid responsive gene were collected from published microarray database; and cutoff was 2 folds (Moran et al. 2002; De Vos et al. 2005; Couldridge et al. 2007; Kuśnierczyk et al. 2007; Kuśnierczyk et al. 2008; De Vos & Jander 2009). To find target genes displayed different expressed patterns in *ago4* and wild-type plants after GPA infestation, among these 127 genes, 95 genes were selected based on the existing of DNA methylation sites. Then, gene expression patterns of 95 genes were analyzed in *ago4* and wild-type with GPA infestation. As shown in Table 2, ten of them displayed the different gene expression pattern between *ago4-1* and *clk-st* after aphid's infestation. which indicated that these genes were likely to be regulated by AGO4. They are AUXIN RESISTANT 3 (AXR3), COPPER/ZINC SUPEROXIDE DISMUTASE 1 (CSD1), CYP79F1, PLDGAMMA1,

CYTOCHROME P450 MONOOXYGENASE 83B1, TRYPTOPHAN BIOSYNTHESIS 1 (*TRP1*),ANKYRIN REPEAT FAMILY PROTIEN, FRUCTOE-1,6-BIPHOSPHATASE FAMILY PROTEIN, ARABIDOPSIS THALIANA GIBBERELLIN 20-OXIDASE 1, PHENYLALANINE AMMONIA-LYASE 2. In addition, some genes such as ankyrin repeat family protein, which displayed heavy DNA methylation pattern in its promoter region, which indicated that this gene is likely to be regulated by DNA methylation.

Table 1. Aphids' Performance on *Ago4-1* and Wild-type Plants.

	Nymphal development, hours[1]				Survival rate (%) of numph[2]	Aphid fecundity, nymphs/day[3]	Longevity, days[4]
	1st instar	2nd instar	3rd instar	4th instar			
Ler	36.3± 2.0 a	39.5±0.8 a	36.5±1.2 a	55.9±1.6 a	97.9	2.8 ± 0.1 ab	19.0 ± 0.5 a
clk-st	37.0 ± 2.4 a	39.5± 1.8 a	37.2±2.4 a	56.1± 2.9 a	95.8	2.5 ± 0.2 b	19.1 ± 0.6 a
ago4-1	36.7 ± 0.6 a	38.6 ±0.8 a	36.4±2.3 a	49.1± 1.8 b	95.8	3.2 ± 0.3 a	18.5 ± 1.1 a

All experiments were repeated four times. Each time were performed on 12 plants per genotype. The data sets were combined all the replicates. Column means followed by the same letter are not significantly different from each other.

[1] Mean developmental time needed (± standard error) for every new-turned nymph to develop into next instar

[2] Overall survival rate of nymph from new hatch to adult

[3] Mean progeny (± standard error) of single adult apterous aphids hatch daily

[4] Mean longevity (± standard error) of adult apterous aphids*

(Wei N unpublished data)

III.4 Aphid Bioassay on Mutants of 25 Selected Genes

In order to study the biological function of 25 selected genes, and to study the function of AGO4 in plant resistance against aphids, we performed bioassay test on 38 T-DNA mutant lines of these 25 selected genes. As shown in Table 3, some mutant lines displayed different phenotype compared to wild-type plant during GPA infestation. For example, in no-choice test, two T-DNA mutant lines of *TRP1* are preferred to be selected by GPA; both T-DNA mutant lines displayed the same pattern of aphids' preference, which indicated that *TRP1* was likely to be an aphid resistant gene (Figure 2).

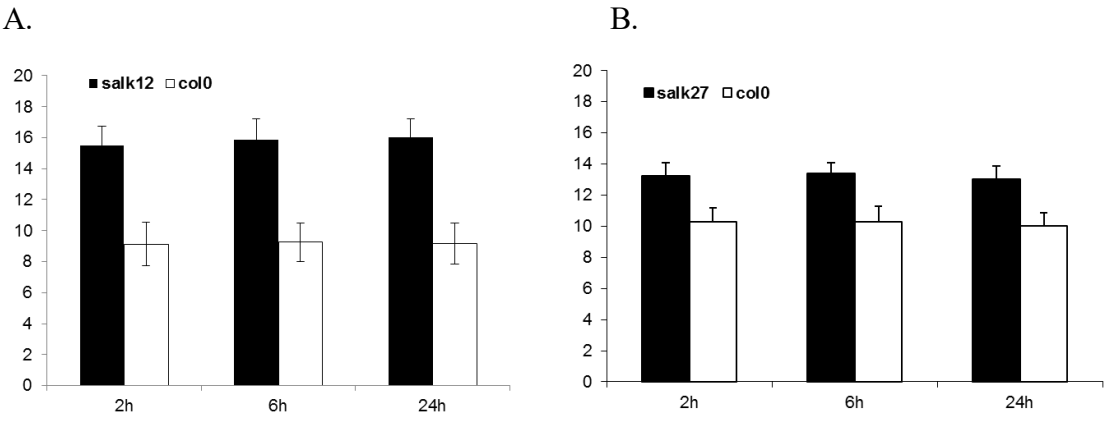

Figure 2. GPA Choice Test on *trp1* Salk line Mutant Plants.
Salk12 and salk27 are two T-DNA mutants of *TRP1* gene.
In Figure A, P value is 0.00 at 2h, 6h and 24h between salk12 and col-0.
In Figure B, P value is 0.05 at 2h, 6h, and 24h between salk27 and col-0.

Table 2. 25 Selected Genes List.

Accession Number	Gene Name	Expression pattern	
		clk-st	*ago4-1*
At1g02050	chalcone and stilbene synthase family protein	↑	↑
AT1G04250	AXR3 (AUXIN RESISTANT 3)	↑	↓
AT1G08830	CSD1	↑	↓
AT1G16410	CYP79F1, CYTOCHROME P450 79F2	↑	↓
At1g25230	purple acid phosphatase family protein	↓	↓
AT1G71880	SUCROSE-PROTON SYMPORTER 1	↑	↑
At1g74100	desulfoglucosinolate sulfotransferase (SOT 16)	↓	↓
AT2G02990	RNS1	↑	↑
AT2G14610	PATHOGENESIS-RELATED GENE 1	-	-
AT3G01420	alpha-dioxygenase 1	↑	↑
AT3G05730	Encodes a defensin-like (DEFL) family protein	↓	↓
AT3G53260	PHENYLALANINE AMMONIA-LYASE 2	-	↑
AT3G61990	O-methyltransferase	↑	↑
AT4G11850	PLDGAMMA1	↑	↓
AT4G17500	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1	↑	↑
AT4G18170	WRKY DNA-BINDING PROTEIN 28	↑	↑
At4g25420	ARABIDOPSIS THALIANA GIBBERELLIN 20-OXIDASE 1	-	↓
At4g31500	CYTOCHROME P450 MONOOXYGENASE 83B1	↑	↓
At5g01600	ATFER1	↑	↑
AT5G17990	PHOSPHORIBOSYLANTHRANILATE TRANSFERASE	↑	↓
At5g25610	RD22	↓	↓
AT5G54710	ankyrin repeat family protein	↓	↑
AT3G01420	ALPHA-DOX1 (ALPHA-DIOXYGENASE 1)	↑	↑
AT5G64380	fructose-1,6-bisphosphatase family protein	↑	↓
AT1G62510	lipid transfer protein (LTP) family protein)	↑	↑

"↑" means up-regulation by GPA infestation; "↓" means down-regulation by GPA infestation, "–"means no change

Table 3. Summary of Bioassay Results.

No.	Accession No.	gene name	Insertion	Choice test	Non-Choice test
1	At1g02050	POLYKETIDE SYNTHASE A	UTR	N	N
2	At1g02050	POLYKETIDE SYNTHASE A	Intron		
3	AT1G04250	AUXIN RESISTANT 3	1st Exon	N	N
4	AT1G04250	AUXIN RESISTANT 3	1st Intron	N	Y
5	AT1G08830	COPPER/ZINC SUPEROXIDE DISMUTASE 1	3rd Exon	N	N
6	AT1G08830	COPPER/ZINC SUPEROXIDE DISMUTASE 1	1st Intron	N	Y
7	AT1G16410	CYTOCHROME P450		N	N
8	AT1G16410	CYTOCHROME P450	1st Exon	N	N
9	At1g25230	unknown	promoter	Y	N
10	AT1G71880	ARABIDOPSIS THALIANA SUCROSE-PROTON SYMPORTER 1	promoter	N	N
11	AT1G71880	ARABIDOPSIS THALIANA SUCROSE-PROTON SYMPORTER 1	promoter	Y	N
12	At1g74100	ARABIDOPSIS SULFOTRANSFERASE 5A	Exon	N	N
13	AT2G02990	RIBONUCLEASE 1	Promoter	N	N
14	AT2G14610	PATHOGENESIS-RELATED GENE 1 (PR1)		N	N
15	AT2G14610	PATHOGENESIS-RELATED GENE 1 (PR1)	Promoter		
16	At3g01420	ALPHA-DIOXYGENASE 1		Y	N
17	At3g01420	ALPHA-DIOXYGENASE 1		N	N
18	AT3G05730	unknown		N	N
19	AT3G05730	unknown	Promoter	Y	N
20	AT3G53260	PHENYLALANINE AMMONIA-LYASE 2		N	Y
21	AT3G53260	PHENYLALANINE AMMONIA-LYASE 2		N	N
22	AT3G61990	O-MTASE FAMILY 3 PROTEIN		N	N
23	AT4G11850	PHOSPHOLIPASE D GAMMA 1	3rd Intron	N	N
24	AT4G11850	PHOSPHOLIPASE D GAMMA 1	7th Exon		
25	AT4G17500	ETHLENE RESPONSIVE ELEMENT BINDING FACTOR 1		N	N
26	AT4G18170	WRKY DNA-BINDING PROTEIN 28	Promoter	N	N
27	AT4G25420	ARABIDOPSIS THALIANA GIBBERELLIN 20-OXIDASE 1	3rd Exon	Y	Y
28	AT4G25420	ARABIDOPSIS THALIANA GIBBERELLIN 20-OXIDASE 1	2nd Intron		
29	At4g31500	CYTOCHROME P450	UTR	Y	N
30	At4g31500	CYTOCHROME P450	2nd Exon	N	N
31	At5g01600	ARABIDOPSIS THALIAA FERRETIN 1	7th Exon	N	N
32	At5g01600	ARABIDOPSIS THALIAA FERRETIN 1	2nd Intron	Y	N
33	At5g17990	TRYPTOPHAN BIOSYNTHESIS 1	2n Exon	Y	N
34	At5g17990	TRYPTOPHAN BIOSYNTHESIS 1	3rd Intron	Y	N
35	At5g25610	RESPONSIVE TO DESSICATION 22	2nd Intron	Y	N
36	At5g25610	RESPONSIVE TO DESSICATION 22	1st Intron	Y	N
37	AT5G54710	unknown		N	N
38	AT5G54710	unknown	1st Exon	N	N

Note: N indicates no significant differences; Y indicates there is significant difference between mutant and wild-type plants; P value is less than 0.05.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The data presented in this work provide evidence for a role of AGO4 in plant resistance against GPA. Here we studied the functional arena of plant ARGONAUTE4 (AGO4) from molecular function of DNA methylation and sRNA biogenesis to the regulation of traits mediating the direct defense of plants against GPA.

Argonaute proteins are components of plant RdDM mechanisms. In *Arabidopsis*, AGO4 is involved in RdDM, a gene silencing process. In this pathway, siRNAs is processed by RNAse III enzymes DICER-LIKE 3 from related dsRNAs and, which is then loaded to AGO4 protein to form the AGO4-*siRNA* complex, and AGO4 protein recruits the protein DOMAINS REARRANGED METHYLTRANSFERASE 2, which catalyzes *de novo* DNA methylation at corresponded sites, that is involved in plant immunity and defense. The role of AGO genes in plant defense against pathogen and bacterial has been studied for a long time and AGO4 was involved in plant resistant against virulent *Pseudomonas syringae* and the avirulent *P.s.t.* (Agorio & Vera 2007).

In our study, it was shown that *ago4-1* mutant plants were compromised in GPA infestation.

Plant damage assays indicated that the symptom development caused by GPA infestation was more rapid in *ago4-1* mutant than *clk-st* plants (Figure 1). Moreover, there was only 60% chlorophyll contents remained in *ago4-1* mutants after 10 d feeding by GPA, whereas, *clk-st* had 95%. In addition, GPA feeding on *ago4-1* plants showed better performance than those aphids feeding on wild-type plants (Table 1), Time required for fourth instar to adult is 6~7 hours faster of aphids feeding on *ago4-1* mutants than those feeding on Ler and *clk-st* plants (Table 1). Also, adults aphids raised on *ago4-1* displayed higher fecundity than those raised on *clk-st* plants. It has been reported that DNA hypomethylation caused constitutively expression of gene *Ep5C* contributes to the compromised resistancte of *ago4* to *P.s.t. DC3000*. Gene *Ep5C*encodes an extracellular peroxidase that required for *P.s.t. DC3000*susceptibility in *ago4* mutant. Tofurther study molecular mechanism of *ago4* susceptible to GPA, downstream genes both regulated by AGO4 and involved in GPA resistance are expected. Figure 3 shows my proposed hypothesis: In wild-type, upon GPA infestation, GPA positively regulates an unknown factor through AGO4 and then induces the expression of *TRP1* gene, which further increases plant defense against GPA. The mutation of AGO4 blocks GPA-induced *TRP1* expression, which accounts for the susceptible phenotype of *ago4-1* to GPA. Interestingly, it was reported that *AGO4* gene expression was up-regulated in *Gossypium hirsutum L.* (cotton) after *Aphis gossypii*infestation (Dubey et al. 2013).

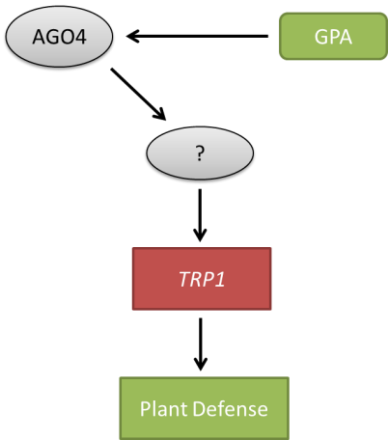

Figure 3. Proposed Model of Mechanism of AGO4 in Plant Defense.

TRP1 a gene involved in tryptophan biosynthesis and tryptophan is the upstream product of indole glucosinolate (IGS) (Iven et al. 2012). It has been reported that glucosinolates as defensive secondary compounds are involved in GPA resistance in *Arabidopsis*(Pfalz et al. 2009). Results of GPA choice-test indicated that *TRP1* gene was likely to be an aphid resistant gene. Moreover, different gene expression pattern of *TRP1* in *ago4-1* and *clk-st* indicated that *TRP1* may be regulated by AGO4. Interestingly, *TRP1* gene in Wassilewskija (WS) strain is densely methylated over the sequence regions at both CG and non-CG cytosines (Luff et al. 1999). DNA demethylation occurs when plants response to bacterial infestation (Yu et al. 2013). Based on the hypothesis that decreased DNA methylation level led to an increased expression level of related genes. It is possible that

DNA methylation level decreased when wild-type plants infested by GPA and resulted in an induction of *TRP1* gene in response to GPA. However, the induction of *TRP1* in *ago4* was not observed and may due to the abolishment of DNA methylation in *ago4* mutants (Figure 4).We concluded that *TRP1* as a GPA resistance gene is likely to be indirectly regulated by AGO4. There might be a transcriptional factor, such as suppressor of *TRP1*, which is directly regulated by AGO4 through DNA methylation. In *ago4*mutant, decreased DNA methylation level resulted in an increased expression of transcriptional factor and then resulted in decreased expression of *TRP1*. Failure to induce the expression of *TRP1* may be responsible for the susceptible phenotype in *ago4* mutant. Further independent qPCR experiments are needed to confirm the different patterns in *ago4* and wild-type plants.

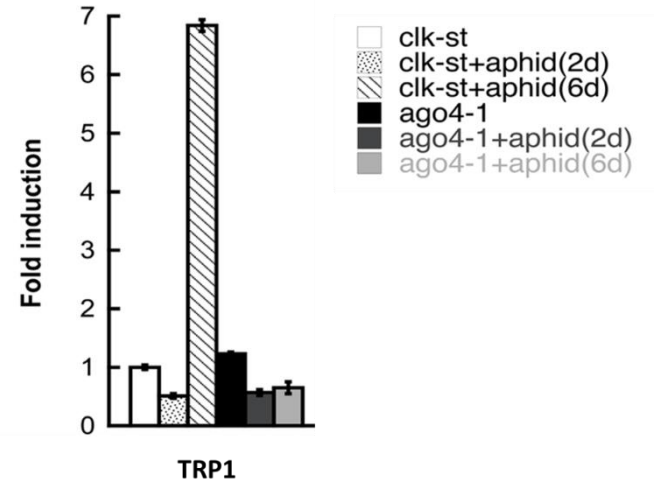

Figure 4. Gene Expression Pattern of TRP1 after GPA Infestation.
Three-week old plants were infested with 30 adult aphids and plant rosettes were harvested at 0, 2 and 6 days after aphids' infestation.

In addition, our study found that another gene, ankyrin repeat family protein contains heavy DNA methylation sites in its promoter region. It has been reported that when promoter region is methylated, gene is negatively regulated by DNA methylation (Chinnusamy & Zhu 2009). Interestingly, expression level of this gene increased in *ago4* mutant (unpublished data) suggesting that this gene direct regulated by AGO4 through DNA methylation. However, no difference between mutant and wild-type plants was observed in bioassay test. Possibly, different experimental conditions could result in different result of bioassay. For example, Graeme J. Kettles et al. (Kettles et al. 2013) found that aphid fecundity increased in the *cyp79b2/ cyp79b3* mutant relative to Col-0.Kim et al. (Kim et al. 2008) found no change in fecundity of aphids raised on *cyp79b2/cyp79b3* mutants relative to Col-0 plants. It is possible that the mutant of ankyrin repeat family protein failed to show any difference of GPA bioassay compared with Col-0 plants. In summary, although no study has been reported about the function of ankyrin repeat family protein in GPA resistance, it is another interesting gene that might contribute to the susceptible phenotype of *ago4* to GPA infestation.

Modification of DNA methylation profiles in response to environmental stresses lead to the transcriptional activation of defense-related genes. The induced defense genes by DNA methylation is proposed to be one of plant immunity mechanisms response to stresses. For

example, the *met1* and the *ddc* mutants were more resistant to bacterial *Pst* DC3000 compared to wild-type. A RNA-seq experiment found that many pathogen-responsive genes were constitutively expressed in *met1* and *ddc* in the basal expression level, e.g. PHYTOALEXIN DEFICIENT4 (PAD4) were up-regulated in *met1* mutant for 2.42 folds and in *ddc* for 4.03 folds (Zhang 2012).

In our study, we found that *ago4* mutant displayed susceptible phenotype response to GPA infestation, which indicated that AGO4 may play an important role in GPA resistance. We also proposed that some GPA resistance related genes, such as *TRP1* were regulated by AGO4. The decreased DNA methylation level in *ago4* mutant could result in a mis-regulation of these defense-related genes. And the expression of defense genes was responsible for the susceptible phenotype of *ago4* mutant to GPA. Although, a proposed gene, such as *TRP1* may be regulated by AGO4 and involved in GPA resistance, however, the direct evidence that *TRP1* is regulated by AGO4 through DNA methylation is needed. Bisulfite sequence can be applied to the future study, to confirm whether DNA methylation level decreases in *TRP1* gene in *ago4* mutant compared to wild-type plants.

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APPENDIX A

SUPPLEMENTARY DATA

Table S1. Information of Salk Lines for Bioassay

Accession No.	Gene Name	Salk line Order	No.	Insertion
At1G02050	CHALCONE AND STILBENE SYNTHASE FAMILY PROTEIN (CSS)	salk_079287C	salk 15	Exon
At1G02050	CHALCONE AND STILBENE SYNTHASE FAMILY PROTEIN (CSS)	SALK_134643C	Salk 26	Exon
AT1G04250	AUXIN RESISTANT 3 (AXR3)	SALK_065697C	Salk 10	Exon
AT1G04250	AUXIN RESISTANT 3 (AXR3)	SALK_011820C	Salk 4	Exon
AT1G08830	SUPEROXIDE DISMUTASE 1 (CSD1)	SALK_109389C	Salk 21	Exon
AT1G08830	SUPEROXIDE DISMUTASE 1 (CSD1)	SALK_024857C	Salk 6	Exon
AT1G16410	CYP79F1 (CYP)	CS870627	Sail 4	Exon
AT1G16410	CYP79F1 (CYP)	SALK_098658	Salk 20	Exon
At1G25230	CALCINEURIN-LIKE PHOSPHOESTERASE SUPERFAMILY PROTEIN (CLP)	SALK_084267C	Salk 16	Intron
AT1G71880	SUCROSE-PROTON SYMPORTER 1 (SPS)	SALK_073498	Salk 14	1000-Promotor
AT1G71880	SUCROSE-PROTON SYMPORTER 1 (SPS)	SALK_123324	Salk 24	1000-Promotor
AT1G74100	DESULFOGLUCOSINOLATE SULFOTRANSFERASE (SOT 16)	SALK_003961	Salk 1	Exon
AT2G02990	RIBONUCLEASE 1 (RNS1)	SALK_087165C	Salk 17	1000-Promotor
AT2G14610	PATHOGENESIS-RELATED GENE 1 (PRG1)	CS874182	Sail 6	Exon
AT2G14610	PATHOGENESIS-RELATED GENE 1 (PRG1)	SALK_148459	Salk 30	1000-Promotor
AT3G01420	ALPHA-DIOXYGENASE 1 (AD1)	SALK_005633C	Salk 2	Intron
AT3G01420	ALPHA-DIOXYGENASE 1 (AD1)	SALK_113614C	Salk 22	Exon
AT3G05730	DEFENSIN-LIKE FAMILY PROTEIN (DEFL)	CS833148	Sail 3	1000-Promotor
AT3G05730	DEFENSIN-LIKE FAMILY PROTEIN (DEFL)	SALK_031670	Salk 7	1000-Promotor
AT3G53260	PHENYLALANINE AMMONIA-LYASE 2 (PAL2)	CS874221	Sail 7	Exon
AT3G53260	PHENYLALANINE AMMONIA-LYASE 2 (PAL2)	SALK_092252C	Salk 18	Intron
AT3G61990	O-METHYLTRANSFERASE (OMF)	CS821745	Sail 2	300-UTR5
AT4G11850	PLDGAMMA1 (PLM1)	SALK_066687C	Salk 11	Exon
AT4G11850	PLDGAMMA1 (PLM1)	SALK_113873	Salk 23	Intron
AT4G17500	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1 (ERE1)	SALK_036267	Salk 8	300-UTR5
AT4G18170	WRKY DNA-BINDING PROTEIN 28 (WDB28)	SALK_007497	Salk 3	1000-Promotor
AT4G25420	ARABIDOPSIS THALIANA GIBBERELLIN 20-OXIDASE 1 (AG20)	CS871868	Sail 5	Exon
AT4G25420	ARABIDOPSIS THALIANA GIBBERELLIN 20-OXIDASE 1 (AG20)	SALK_094207C	Salk 19	Intron
AT4G31500	CYTOCHROME P450 MONOOXYGENASE 83B1 (CP83B1)	SALK_071430C	Salk 13	300-UTR5
AT4G31500	CYTOCHROME P450 MONOOXYGENASE 83B1 (CP83B1)	SALK_012581	Salk 5	Exon
AT5G01600	ATFER1 (ATF1)	SALK_142964	Salk 28	Exon
AT5G01600	ATFER1 (ATF1)	SALK_151384C	Salk 31	Intron
AT5G17990	TRYPTOPHAN BIOSYNTHESIS 1 (TRP1)	SALK_142670C	Salk 27	Exon
AT5G17990	TRYPTOPHAN BIOSYNTHESIS 1 (TRP1)	SALK_069482	Salk 12	Intron
AT5G25610	RESPONSIVE TO DEHYDRATION 22 (RD22)	SALK_146066C	Salk 29	Intron
AT5G25610	RESPONSIVE TO DEHYDRATION 22 (RD22)	SALK_063371	Salk 9	Intron
AT5G54710	ANKYRIN REPEAT FAMILY PROTEIN (ARPF)	CS812168	Sail 1	Intron
AT5G54710	ANKYRIN REPEAT FAMILY PROTEIN (ARPF)	SALK_127497C	Salk 25	Exon

Salk lines were selected based on the available seeds from Arabidopsis Biological Resource Center (ABRC) and the insertion sites of T-DNA on related genes.

Table S2. Primer List of 38 T-DNA Lines.

Accession No.	Salk line number	LP	RP
At1g02050	salk_079287C	CTTCCTGTTGCAACCACTTTC	CTTGTGGATTTGCTGCTTAGC
At1g02050	SALK_134643C	ATGCAACACGGTCATAGAAGG	CTTGATGCAACCTAAACTGGC
AT1G04250	SALK_065697C	CGATTTTCCTCAAGTACGGTG	TTTCCTTCACTTGTGCTTTCG
AT1G04250	SALK_011820C	AAGCAAAGACATTTGACCACG	TGAAGGTATCAATGGACGGAG
AT1G08830	SALK_109389C	TCTTCTGAAGATGCCTTGACC	GTCATTACCCTTTCCGAGGTC
AT1G08830	SALK_024857C	ATGAACCCCGAGTTACCAGAG	TTGCAGTTTTGAACAGCAGTG
AT1G16410	CS870627	GGCGTTTCCTAGTCTTCCATC	TTTTTACGGCTAAAAGACGTTTAC
AT1G16410	SALK_098658	TCCGGGTTCTTTAACATTTCC	GGCATCAATCACTCTACTGGG
At1g25230	SALK_084267C	AAGCAATGATCATGACCGTTC	TGAAAAGTATCGATGATCCCG
AT1G71880	SALK_073498	AGAGAGAGCTGTAAGGCCCAC	TACCATCTTTGGGACTGTTCG
AT1G71880	SALK_123324	GACCACAGAGCCAAATGAGAG	TGTTGCCCTGAACCATCTATC
At1g74100	SALK_003961	ACCAAGCCTCTCTCTACCTGC	CAAAGCTGTCCTCAAGACTCG
AT2G02990	SALK_087165C	TACCGTTGAGCTATCGAATGG	CATCCAAGAAAGTTTCACACG
AT2G14610	CS874182	AACTTTTAAAAAGGCCACATATTTTAC	CAATGGCAAAGCTACCGATAC
AT2G14610	SALK_148459	AGCTTTCGAGGGAAGAACAAG	CAATTTTTCTGATTCGGAGGG
AT3G01420	SALK_005633C	GAATGGGTTGGTTAACCAACC	TGCTAAAAATGGGAAAACGTG
AT3G01420	SALK_113614C	ACCATTCCGATACAACACAGG	GCCTCCGTAACTTCAAAAAGG
AT3G05730	CS833148	CTGAATTCAATTTTCCACCCC	GAAGAGTGGTGAAGCAGATGG
AT3G05730	SALK_031670	GCAATGCTCTTCTTCGTTGTC	ACCACCCCACCCTTGTACTAC
AT3G53260	CS874221	TTGGACATTCGCTTCGAATAG	AACCAAAAACCACCAACAATTC
AT3G53260	SALK_092252C	TTTGCGACTCAAACCAAATTC	AAGAGAGAGGAACGAGATCGC
AT3G61990	CS821745	ACAGGTGAATGATGCGAAGAC	CTTCTCGTCGTCAGCCATAAC
AT4G11850	SALK_066687C	CCTCTGCTGCATACTGTCTCC	TGGGATTCAAACAAGGACTTG
AT4G11850	SALK_113873	ATCCGGCTAATGTTCCGTATC	AGTGCTGAGCAGATCGTATGG
AT4G17500	SALK_036267	CGTTCCTAACCAAACCCTAGC	TCCTACTCTTCTCCCTGCTCC
AT4G18170	SALK_007497	TTTAATTCCCCACAATTATCGC	TGAATGACTTTTTGGTTTCGG
At4g25420	CS871868	GTGCCAGTTTTGAATGAATGG	CGCAGAAGTAATCTTGAACGG
At4g25420	SALK_094207C	TTGGTGAGCCAATCTGAAAAG	TTGATGTTCCTCTCATCGACC
At4g31500	SALK_071430C	TCAAGGAATCCGAAATAAGGG	TGATCGCTTCAAATTTCATAGG
At4g31500	SALK_012581	TGCGAAGTCAATAGGGACATC	GCTCACTTAGATCAACGGTGC
At5g01600	SALK_142964	TGGGGATCATTGTTCTCTGAG	TCTACATTTTCCAACGATGGC
At5g01600	SALK_151384C	TATCCCAGTGACCAGCTTGTC	TCGAACATGCTGAAAAAGGAG
AT5G17990	SALK_069482	TTCGCTTGCTCTCAGAATCTC	TGACATCATAAAACCGATCCC
AT5G17990	SALK_142670C	CCTTTGCTACTTTTGCACCAC	AATCGAGTGATGGTTATTGCG
At5g25610	SALK_146066C	ACCGGACATTCATTTCTTTCC	AGCATTTAAGGAGCAGAAGGC
At5g25610	SALK_063371	AGACCGATTTACGTTGGTGTG	GCGAATGGGTACTTCTGTTTG
AT5G54710	CS812168	TACTTCCTAGCGCAACGCTAG	CAAAGTAGTTGTTGGCATTTGC
AT5G54710	SALK_127497C	AAATGACAATCCAGTGGCAAC	TTTTCAATGCAGTTTTGAAGTC

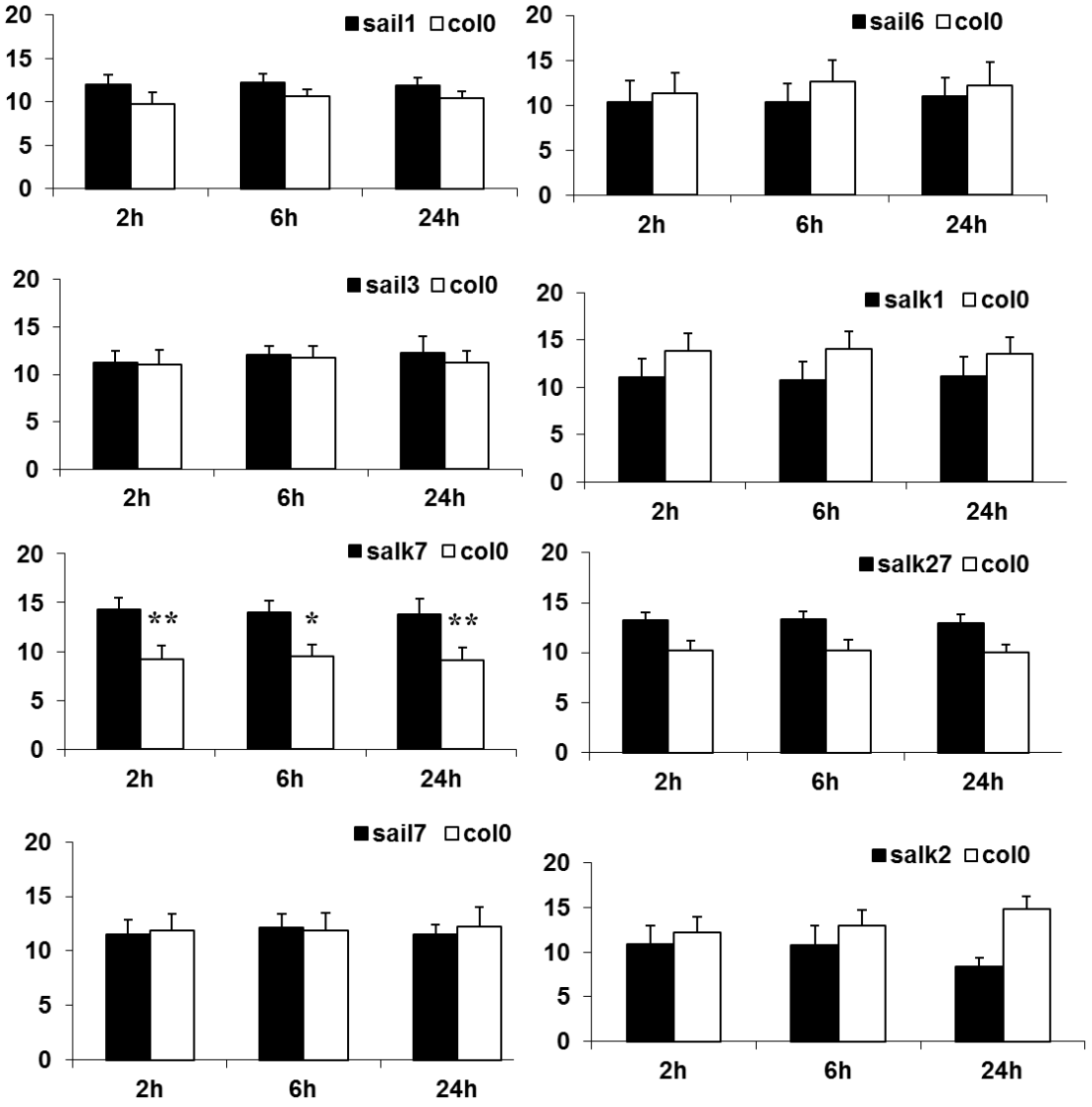


Figure S1. Summary of Choice Test Results of Salk-line Mutants. Vertical axis represents the average aphids' number of each genotype (wild-type and mutant). Horizontal axis represents different time points after releasing aphids between wild-type plant and mutant plant. '*' indicates $0.01 < P \text{ value} < 0.05$. '**' indicates $P \text{ value} < 0.01$.

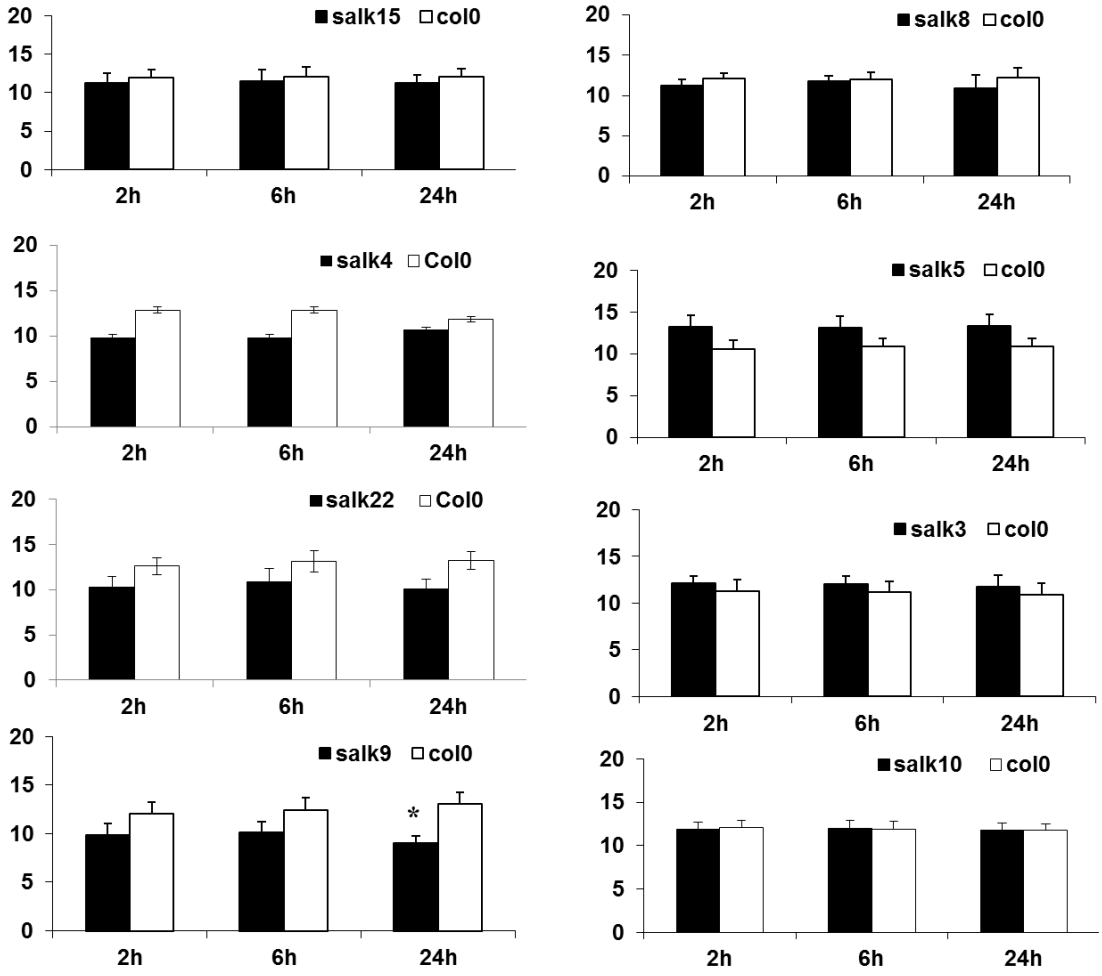
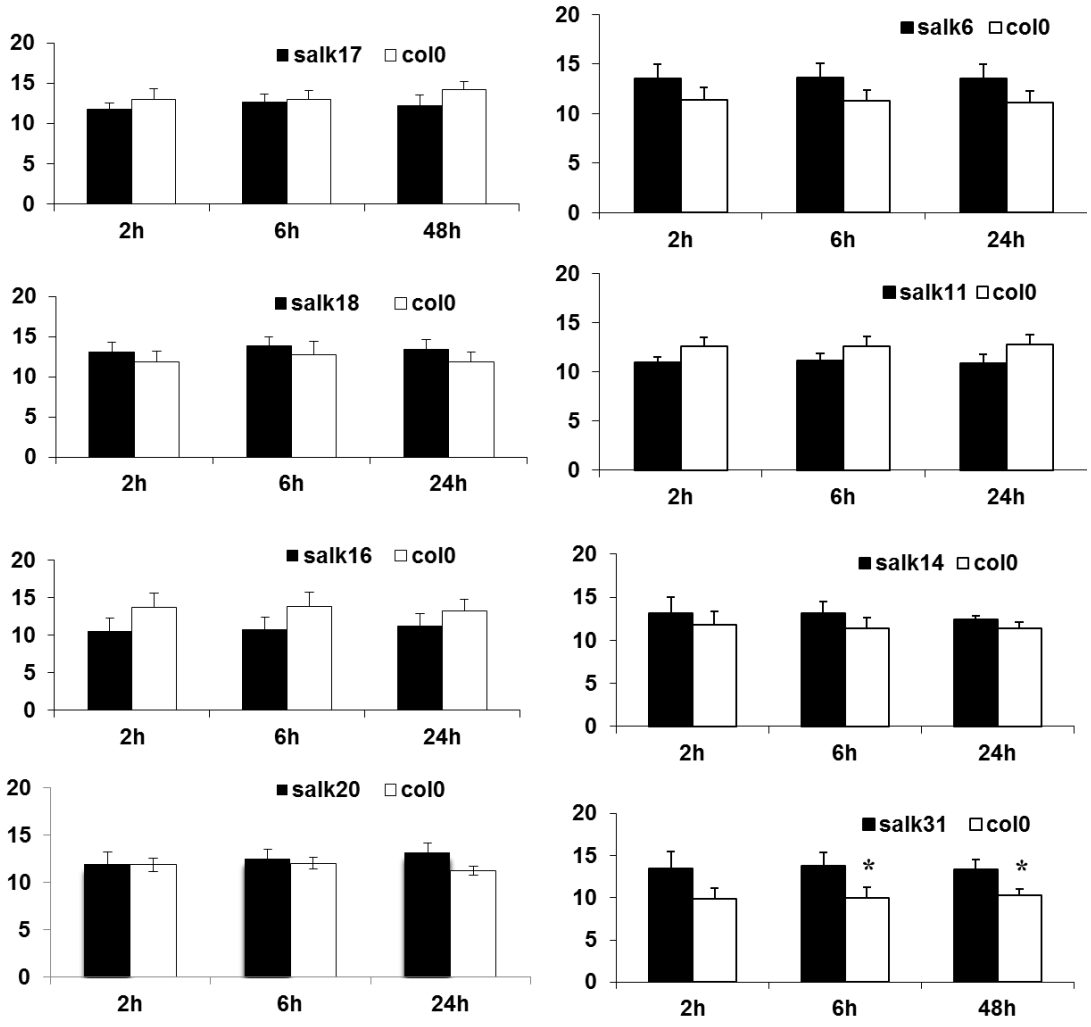


Figure S1. Continued.



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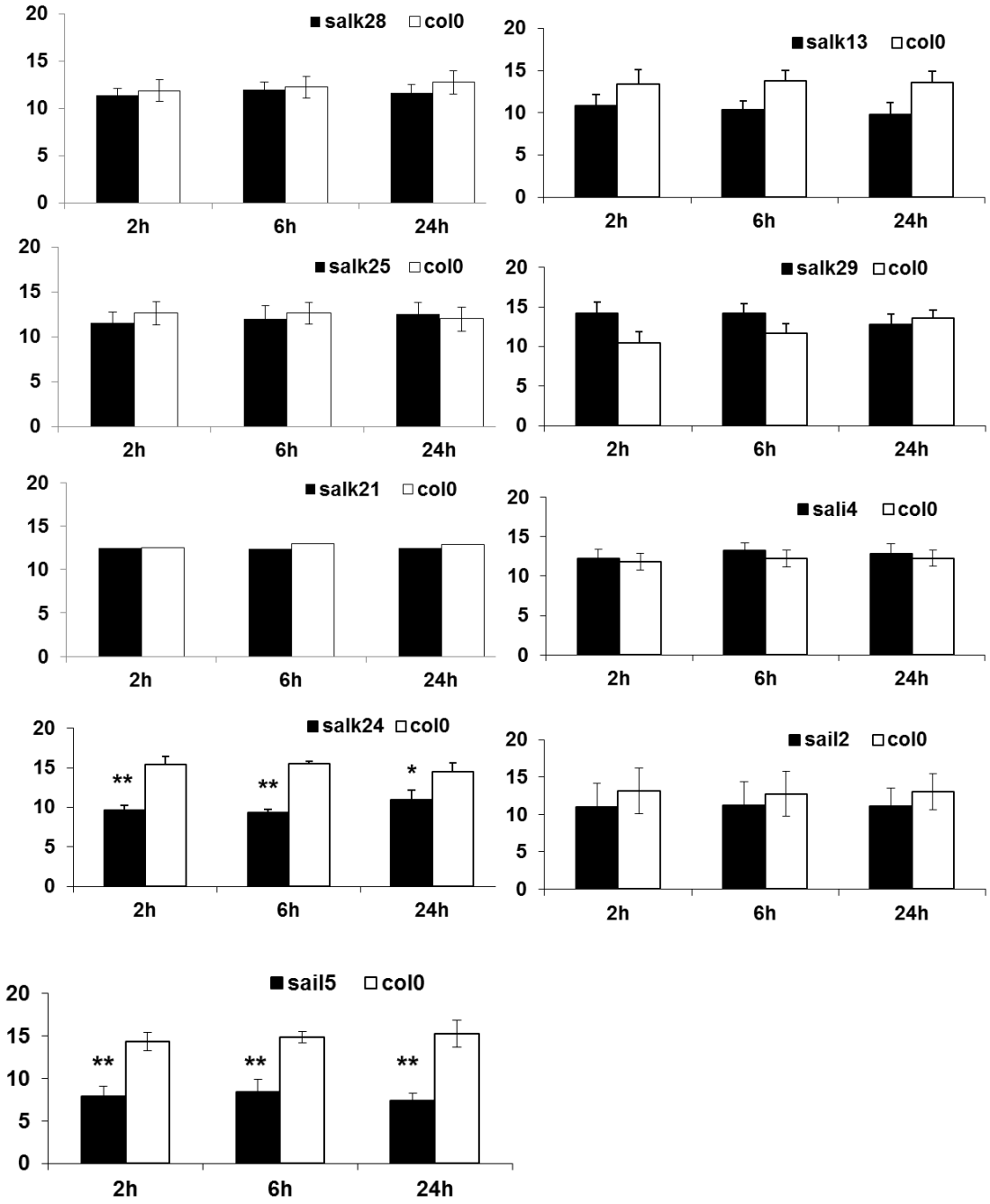

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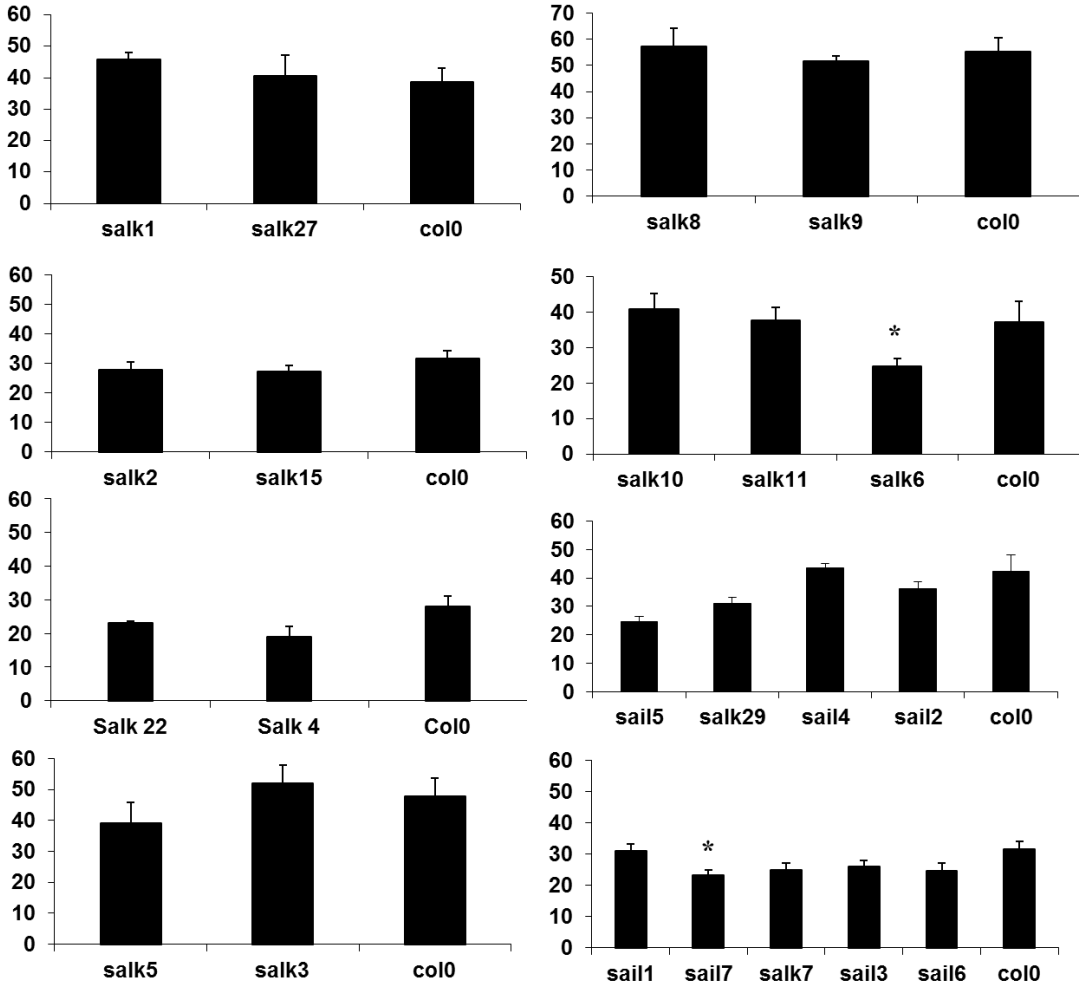

Figure S2. Summary of Non-choice Test Results of Salk-line Mutants. Y-axis represents average total aphids' number on each plant, including adults and nymphs. X-axis represents genotype of plants. '*' indicates 0.01 < P value < 0.05. '**' indicates P value < 0.01.