Genome-wide association study highlights escape from aphids by delayed growth in *Arabidopsis thaliana*

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# Abstract

A growing number of ecological studies have shown that plant phenological and architectural traits modulate herbivore colonization to host plants, but their key genes remain largely unknown. Here, we conducted a genome-wide association study (GWAS) of aphid abundance in a field population of *Arabidopsis thaliana*. Near a single significant peak, we found growth-related genes, such as *ROOT HAIR DEFECTIVE3*, and candidate genes with unknown functions. Out of the unknown genes, a mutant of the putative ribosomal gene (AT3G13882) exhibited far slower growth and later flowering than Col-0 under a long-day condition in a laboratory. Our laboratory experiment further showed that the turnip aphid *Lipaphis erysimi* failed to colonize on the mutant of AT3G13882 that had approximately 60% smaller size than the wild type. These findings suggest that the side effects of growth-related genes on herbivore abundance may be more important than currently appreciated.

# Introduction

Plants are attacked by herbivores across their life cycle in natural environments. While chemical and physical traits have long been a main focus of anti-herbivore defense [1], plant life-history traits also account for herbivory in field environments [2,3]. For example, phenological changes can allow plants to escape from seasonal herbivory [4,5]. Plants’ visibility for herbivores, namely plant apparency [6], also changes from vegetative to reproductive phase, which alters the risk of herbivore attacks across plant ontogeny [3,7]. By focusing on intraspecific variation within a plant species, several studies have shown that plant phenological and architectural traits shape heritable variation in herbivory among plant genotypes [8–10]. Yet, only a few key genes have been identified so far [11].

Genome-wide association study (GWAS) provides a powerful approach to dissect the genetic architecture of ecologically important traits [12,13] and identify novel genes from natural phenotypic variation [14,15]. However, controlled laboratory conditions unlikely reflect outdoor environments where interspecific interactions typically occur [16,17], highlighting the importance of *in natura* study on gene functions [18–21]. For *in natura* understandings of functional genes involved in interspecific interactions, it is necessary to conduct GWAS under field conditions.

*Arabidopsis thaliana* is a model plant species distributed throughout Europe and naturalized around the world. While *A. thaliana* usually bloom in spring after over-wintering, some cohorts have overlapping life cycles from spring to autumn [21–23]. When plants emerge from late spring to early summer, they are threatened by various herbivores [10,24]. Of the diverse insect herbivores, aphids are known as a major herbivore occurring across a natural distribution range of *A. thaliana* [25]. Because aphids often suck phloem saps from flowering stems, we hypothesized that plant life-history traits may play a key role in harboring aphids under field conditions.

To reveal the genetic architecture of aphid herbivory, we here combined GWAS and mutant analysis in *A. thaliana*. We first conducted GWAS of aphid abundance on 196 *A. thaliana* accessions grown in a field site of Zurich, Switzerland. We observed several known genes involved in delayed growth, in addition to candidate genes with unknown functions, near a peak of GWAS. To further test the candidate genes, we then cultivated and released the turnip aphid *Lipaphis erysimi* on *A. thaliana* mutants.

# Materials & Methods

## Field GWAS

### Plant genotypes

We obtained *Arabidopsis thaliana* genotypes that were selfed and maintained as inbred lines, called “accession”, from the Arabidopsis Biological Resource Center (<https://abrc.osu.edu/>). We used 196 *A. thaliana* accessions from RegMap [26] and 1001 Genomes [27] projects, most of which were overlapped with previous GWAS of biotic interaction [28,29]. The list of accessions and phenotypes measured in this study is available in Table S1.

### Field experiment

To observe aphids on a simulated late cohort, we exposed the *A. thaliana* accessions to field environment from 4 to 25 July in 2018 at a field site within Europe. We initially cultivated *A. thaliana* in a laboratory under a short-day condition in order to keep all the plants vegetative at the start of the field experiment. Seeds were sown on 33-mm diameter Jiffy-seven(R) pots and stratified under a constant dark 4C condition for a week. Seedlings were grown for 6 weeks under 8L:16D cycle with 20C air temperature. Plants were then potted in a plastic pot filled with mixed soils of agricultural composts (Profi Substrat Classic CL ED73, Einheitserde Co.) and perlites with a compost to perlite ratio of 3:1 litter volume. Eight replicates of the 196 accessions were then transferred to the outdoor garden at the University of Zurich-Irchel (Zurich, Switzerland; 47 23’N, 8 33’E). Aphids were counted by a single observer every two or three days. To examine whether the aphid abundance differed between plants with and without flowering stems, we also recorded the presence or absence of bolting two weeks after the start of field experiment.

### Data analysis

GWAS was performed using the GWA-portal website (<https://gwas.gmi.oeaw.ac.at>) [30]. The target phenotype was the maximum number of any aphids per plant throughout the experiment. The imputed full-sequence dataset were chosen as SNP data for the 196 accessions. Pseudo-heritability was calculated for the target phenotype before association mapping. Accelerated mixed models were used for association mapping with a correction of kinship structure. The genome-wide significance level was given at with Bonferroni correction of multiple testing. The number of aphids was log()-transformed to improve normality. Input phenotype data are available as a supplementary material. After the association mapping, candidate genes were searched within ca. 10 kb near a focal SNP.

## Mutant analysis

### *Arabidopsis thaliana* mutants

T-DNA sequence-indexed lines of *A. thaliana* was obtained from the Nottingham Arabidopsis Stock Centre (NASC) (<https://arabidopsis.info/>). In addition to Columbia-0 (Col-0) wild type, we ordered four mutant lines per gene for three candidate genes, *MYB26* (AT3G13890), *EPFL3* (AT3G13898), and a putative ribosomal gene (AT3G13882) (Table S2). Following the instruction [32], we checked the T-DNA insertion site of the mutant lines by PCR amplification and Sanger sequencing (see Table S2 and S3 for primer information). The mutant lines were back-crossed to the Col-0 wild type for three generations. Because some lines failed to germinate or had no mutations on the exon region during the back-crossing and confirmation, we finally obtained one confirmed line for each gene: SALK\_112372.30.80.x (NASC Accession ID: N612372) for *MYB26*; SAIL\_1220b\_A10 (N844563) for *EPFL3*; and SALK\_039481.29.99.f (N670586) for AT3G13882. All the three lines carried the T-DNA insertion on the exon (and a part of intron) region of the target gene.

### Laboratory experiments

To observe plant growth, we cultivated 7 - 10 replicates of the three back-crossed mutant lines and the Col-0 wild type under a long-day condition (16h light/8h dark, 22C/20C air temperature). The mutant of *MYB26* gene was sterile due to the lack of anther dehiscence [33]. The pollen was broken physically and self-crossed to stigma by hand. This resulted in the small number of seeds harvested; thus, only seven replicates were prepared for this line. Seeds were sown on an 8 cm pot filled with the agricultural composts (Profi Substrat Classic CL ED73), and stratified at 4 C under a constant dark condition for a week. The stratified seeds were then transferred to the long day condition. Seedlings were grown for 20 days. The rosette diameter (cm) was recorded as an index of plant size before aphids were released as described next. Days to flowering i.e., flowering time was also recorded during the aphid experiment as described next.

To assess the likelihood of aphid colony establishments on the mutant plants, we released the turnip aphid *L. erysimi* on the wild type and mutants of *A. thaliana*. The potted plants were separately enclosed with a mesh net. Five wingless individuals of the turnip aphid *L. erysimi* were released on each plant. The experimental aphids were derived from a source population established by a previous study [11]. The enclosed plants were incubated under the long-day condition. The number of aphids per plant was counted by eyes 3, 7, 10, and 14 days after the release of aphids. We did not count aphids that escaped outside the area of a plant.

### Data analysis

We used generalized linear models (GLM) to test phenotypic differences between each mutant and the Col-0 wild type. Multiple comparisons were corrected using the Bonferroni method. The plant size and flowering time were analyzed using GLMs with Gaussian distribution, which were equivalent to standard linear models. The number of aphids i.e., the count response was analyzed using GLMs with Poisson error structure and a log link function. Wald-tests were used to calculate -values from GLMs.

# Results

## Field GWAS of the aphid abundance

To monitor aphid abundance and visible plant traits, we transplanted 196 *A. thaliana* accessions in the field. The two species of specialist aphids, *Lipaphis erysimi* and *Brevicoryne brassicae*, mainly occurred on *A. thaliana*. Starting from vegetative phase, 38% of individual plants initiated bolting two weeks after the transplant. The total number of aphid individuals throughout the season was more abundant on bolted accessions than on non-bolted accessions (non-bolted and bolted plants = 0 and 7 aphids in median, respectively; Mann-Whitney’s -test, ), indicating that the presence of flowering stem was significantly associated with the aphid abundance. In addition, we also distinguished the abundance of winged and wingless aphids in order to infer ecological processes behind the aphid colonization on *A. thaliana*. Winged and wingless aphids initially colonized vegetative plants three days after the transplant, but many of these aphids did not establish a colony in subsequent monitoring (the days between 07 and 10 July 2018: Fig. S1). This additional observation suggests that colonized aphids do not always establish a colony and thereby success of the colony establishment also depends on the host suitability after colonization.

The total number of aphids had high heritability among the plant accessions (), indicating that this trait was likely under genetic control and thus deserved further GWAS analyses. To detect SNPs strongly associated with the aphid number, we then performed GWAS using the accelerated mixed model. Regarding the aphid number, we detected a significant SNP above the genome-wide Bonferroni threshold (chr3-4579292, , MAF=0.026: Fig. [1](#fig:ManPlot)) in an intergenic region. When comparing trait values between two alleles on the significant SNP marker, three of five accessions shared similar haplotypes from AT3G13870 to AT3G13890 locus (Fig. [S1](#fig:1001browser)), spanning within a 10-kb kbp region. Two growth-related genes were located near this genomic region: AT3G13870 locus, also known as *ROOT HAIR DEFECTIVE3* (*RHD3*), is known to regulate root hair developments [34] and thereby results in delayed growth [35]. AT3G13880 (*OTP72*) locus itself has no visible phenotype, but one allele *otp72-2* is known to affect gene the expression level of *RHD3* [36]. In addition, AT3G13890 locus is known to encode MYB26 transcription factor responsible for the anther dehiscence and male sterility [33]. Known functions of these candidate genes led us to further hypothesize that genes involved in growth or reproduction have significant side effects on aphid colonization on a plant stem.

## Mutant growth and aphid colonization in the laboratory

A putative ribosomal gene (AT3G13882), *EPIDERMAL PATTERNING FACTOR LIKE 3* (*EPFL3*: AT3G13898), and *MYB26* were located nearby the significant SNP (chr3-4579292). We therefore cultivated single-gene mutants of these three genes to examine their visible phenotypes, including growth rate and flowering time. After 20 days of growth period, the rosette size of the AT3G13882 mutant became 3.29 cm on average, which was significantly smaller than wild type plants having 5.38 cm on average (adjusted by Gaussian GLMs: Fig [2](#fig:mutant)A,B). The other two mutant plants were slightly larger than the wild type (6.29 cm and 6.3 cm for *EPFL2* and *MYB26*, respectively), where the *EPFL3* mutant had a significantly larger size than the wild type (adjusted : Fig [2](#fig:mutant)A,B). Consistent with these differences of the initial size, the AT3G13882 mutant took 38.8 days on average until flowering, which was significantly longer than 30.8 days to flowering for the wild type (adjusted : Fig [2](#fig:mutant)A,C). Corresponding to the larger initial size than the wild type, the *EPFL3* and *MYB26* mutants showed earlier flowering than the wild type plants (27.5 days and 23.57 days for *EPFL3* and *MYB26*, respectively: adjusted : Fig. [2](#fig:mutant)D).

The growth difference among the three mutants finally led us to test whether delayed growth could prevent the establishment of aphid colony after colonization. To examine the establishment process after colonization, we released wingless individuals of *Lipaphis erysimi* on the mutant lines besides the wild type of *A. thaliana*. One week after the release, aphids were more likely to failed colonizing the AT3G13882 mutant than on the wild type (adjusted by Poisson GLMs), while *EPFL3* and *MYB26* mutants hosted similar or larger numbers of aphids comparing to wild type plants (Fig [2](#fig:mutant)D). The failure of colony establishments during the former period led to the same patterns until 14 days after the release (Fig [S3](#fig:aphid_stat)), though the levels of statistical significance became larger near the end of experiment likely because over-dispersion of the aphid numbers became severe.

# Discussion

Our field GWAS and laboratory experiment together highlight genetic architecture underlying the side effects of plant life-history traits on herbivore abundance. It is widely recognized that plant genetic variation governs herbivore abundance and communities [8–10], but a keystone gene shaping the ecological communities was not identified until recently [11]. Barbour et al. [11] have experimentally shown that side effects of a glucosinolate biosynthesis gene *AOP2* on plant growth alter *A. thaliana*’s capacity to harbor aphids and their parasitoids. Although we did not detect *AOP2* near the GWAS peak, our study supports the notion that genes associated with plant growth can structure populations or communities of associated organisms.

While ribosomal genes have long been considered housekeeping genes of the protein synthesis machinery, mutants of ribosome-related genes exhibit a wide variety of growth and reproductive phenotypes. A growing number of studies have reported the reduction of leaf cell number [37], reduced root length [38], and the reduction of pollen number [15,39] regarding ribosomal gene mutations. Our findings from the putative ribosomal gene, AT3G13882, adds insights into biotic interactions besides the growth deterioration.

Other than the putative ribosomal gene, mutants of *EPFL3* and *MYB26* exhibited moderate phenotypes of early growth and flowering compared to the wild type. EPFL3 is one of EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) family peptides which are known to control stomatal patterning [40], leaf morphogenesis [41], inflorescence development [42], though biological roles of EPFL3 are still unknown. *MYB26* is more likely to affect flowering than growth (Fig. [2](#fig:mutant)B,C), because this gene is known to encode a transcription factor specifically expressed in a reproductive organ [33]. It was thus possible that mutants of these two genes exhibit the altered phenotypes of growth, flowering, and resultant aphid abundance, though the phenotypes of these two mutants were not so severe as those of the ribosomal gene AT3G13882 (Fig. [2](#fig:mutant)D).

Overall, our study suggests the importance of growth-related genes in driving plant-herbivore interactions in the field. However, we are still unsure whether T-DNA mutation on the putative ribosomal gene (AT3G13882) can represent natural variants responsible for the delayed growth and reduced aphid abundance. Regarding another ribosomal gene *REDUCED POLLEN NUMBER1* (*RDP1*), previous studies showed pleiotropy of this gene on plant growth and pollen number in *A. thaliana* [15,39]. Natural alleles of *RDP1* could alleviate pleiotrophic growth defects [15]. The other growth-related genes or other mutations of AT3G13882 might have reduced the aphid abundance in our field study. Further experimental tests, such as quantitative complementation [15], will be needed to identify natural causal variants that modulate the aphid abidance via delayed growth.

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# Data avaiability

The data and codes are available at the GitHub repository (<https://github.com/yassato/AraAphidGWAS>). The published version is deposited on Zenodo or Dryad (<doi::xxxxxx>).

# Competing interests

The authors declare no conflicts of interests concerning this study.

# Tables & Figures

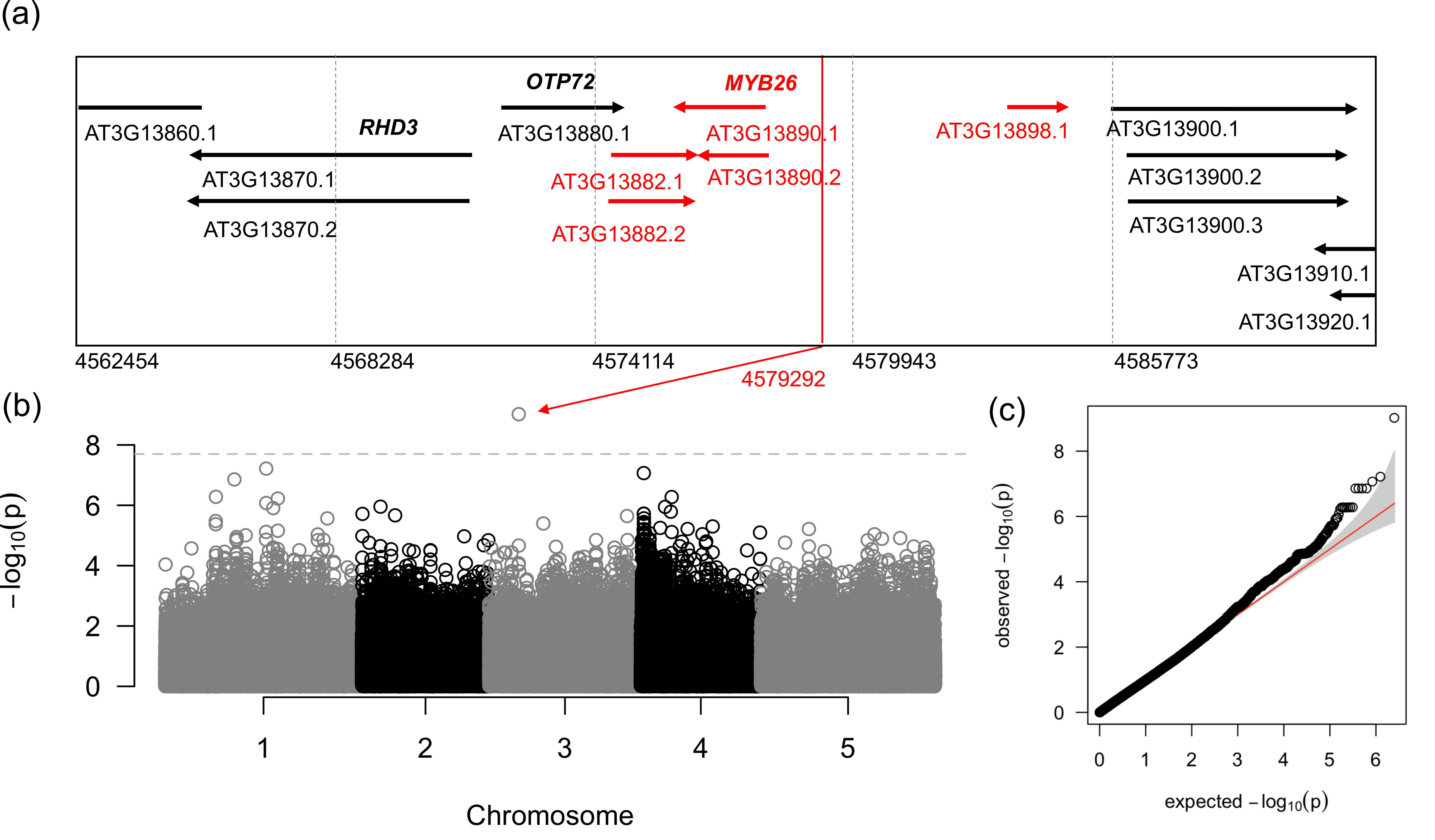


Figure 1. GWAS of the aphid abundance on 196 *A. thaliana* accessions grown in the field. Manhattan plot (b) shows the associaiton score of -log() against five choromosomes of *A. thaliana* at MAF cut-off = 0.025, where a horizontal dashed line indicates the genome-wide Bonferroni threshold at . QQ-plot (c) shows relationships between the observed and expected -log() values. A solid line indicates randomly expected -log() and the shaded area corresponds to its 95% confidence intervals. The upper panel (a) focuses on the top-scoring SNP at Chr3-4579292 and desplays the position of candidate genes.

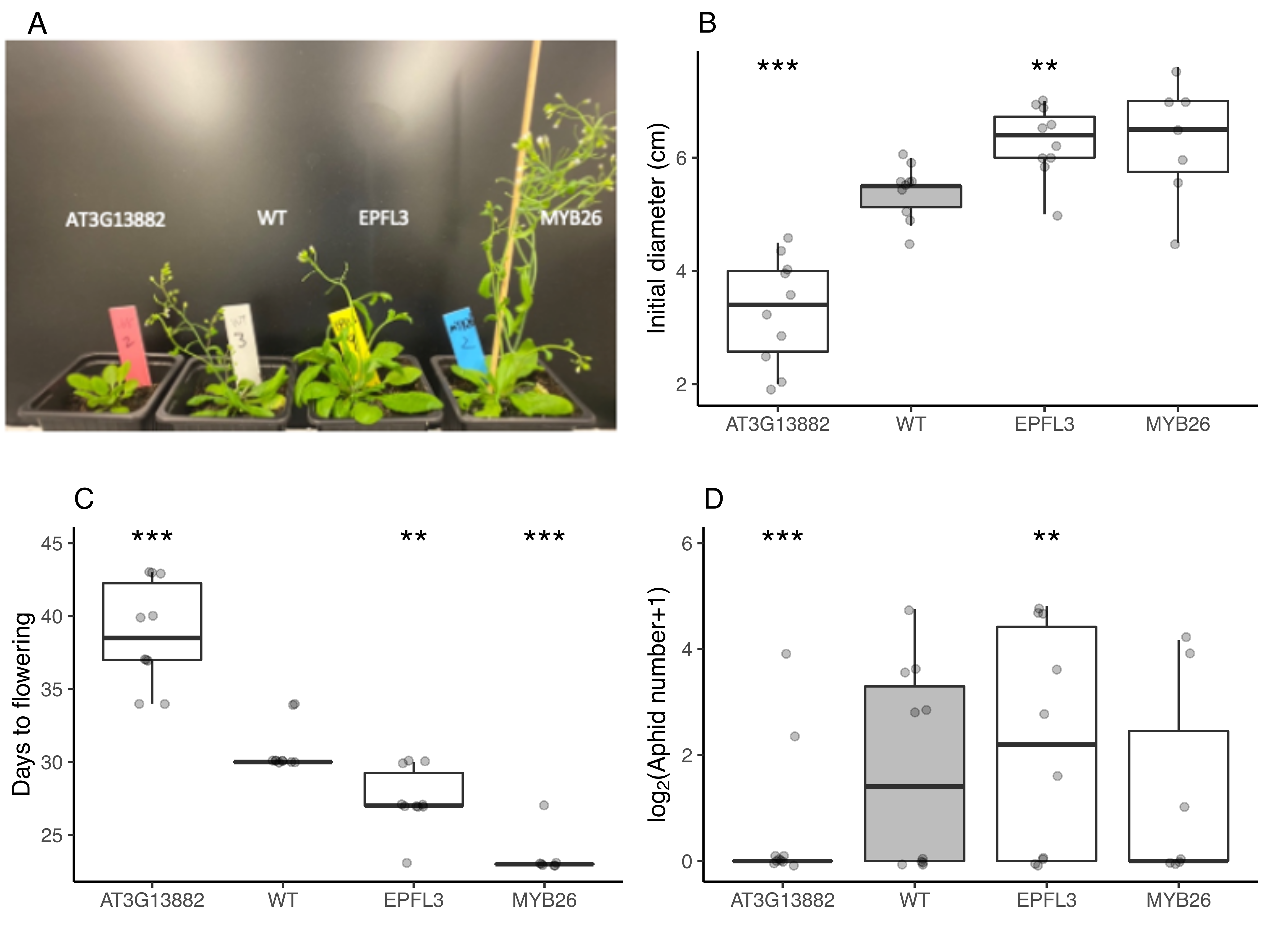


Figure 2. The Col-0 wild type and three mutants of *Arabidopsis thaliana* (A) showing the phenotypes of initial size (B), flowering time (C), and aphid colonization one week after the release (D) in a laboratory. Asterisks above each mutant indicate adjusted -values by GLMs in comparison with the wild type, WT; \*\*\* , \*\* , \* . Boxes: median with upper and lower quartile; Whiskers: 1.5 inter-quartile range.

# Supplementary Materials

![Figure S1. The emergence of wingless and winged aphids on Arabidopsis thaliana accessions grown in the field.](data:application/pdf;base64,)

Figure S1. The emergence of wingless and winged aphids on *Arabidopsis thaliana* accessions grown in the field.

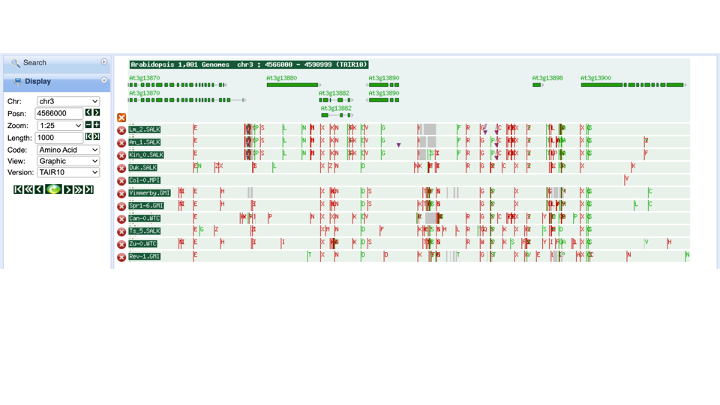


Figure S2. A snapshot of the genomic region near Chr3-4579292 in the 1001 Genome Browser (<http://signal.salk.edu/atg1001/3.0/gebrowser.php>). The upper four accessions carried a rare allele susceptible to aphids (inset of Fig. [1](#fig:ManPlot)). The accessions listed below Col-0 carried a major allele and harbored no aphids in the field GWAS.

![Figure S3. The number of aphids during the later period of incubation. Asterisks indicate significant difference between each mutant and the wild type with Poisson GLMs: *** p<0.001, ** p<0.01, * p<0.05. Boxes: median with upper and lower quartile; Whiskers: 1.5 \times inter-quartile range.](data:application/pdf;base64,)

Figure S3. The number of aphids during the later period of incubation. Asterisks indicate significant difference between each mutant and the wild type with Poisson GLMs: \*\*\* , \*\* , \* . Boxes: median with upper and lower quartile; Whiskers: 1.5 inter-quartile range.

Table S1. List of GWAS accessions and phenotypes.

Table S2. List of mutant lines examined in this study. Red text highlight three-times backcrossed lines.

Table S3. List of primers to check the T-DNA insertion. The primers were designed using T-DNA Primer Design (<http://signal.salk.edu/tdnaprimers.2.html>).

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