

Natural variation in *Arabidopsis thaliana* susceptibility to *Tetranychus urticae* reveals a role for the plastid invertase AtINVE

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Abstract

Background

Natural genetic variation contributes substantially to differences in plant susceptibility to herbivores. Although *Arabidopsis thaliana* responses to herbivory have been extensively studied, the mechanisms underlying variation in susceptibility to the two-spotted spider mite (*Tetranychus urticae*, TSSM) remains poorly understood.

Results

Using a high-throughput phenotyping approach, we quantified susceptibility to TSSM across 134 natural *A. thaliana* accessions and observed substantial variation in leaf damage and feces production. Genome-wide association study (GWAS) identified loci associated with damaged area, pointing to candidate genes involved in metabolic and regulatory processes. Among these, *AtINVE*, encoding a plastid-localized alkaline/neutral invertase, emerged as candidate linking carbohydrate metabolism with susceptibility.

Functional analysis revealed that the *inve-1* knockdown mutant exhibited increased susceptibility to TSSM, supporting a protective role for sugar metabolism during herbivore attack. Comparative analyses of contrasting accessions showed that *AtINVE* expression was higher in the least susceptible accession and decreased following infestation, suggesting possible suppression during herbivory. Although bulk sugar levels and total invertase activity were broadly similar between genotypes, infestation triggered coordinated increases in vacuolar and cell wall invertase activities. These findings indicate that susceptibility is not determined by absolute carbohydrate levels, but rather by dynamic regulation of carbon allocation and metabolic signaling.

Notably, several candidate genes identified by GWAS encode chloroplast-targeted proteins, highlighting chloroplasts as central hubs integrating metabolism and defense responses during herbivore attack.

Conclusions

Our results indicate that susceptibility to TSSM in *A. thaliana* is regulated by interconnected defense and metabolic pathways. We identify *AtINVE* as a novel component linking sugar metabolism to plant defense and highlight the importance of chloroplast-associated processes in modulating herbivore susceptibility. This work provides a framework for understanding how natural variation in carbon metabolism contributes to plant-herbivore interactions.

Background

Plants, as sessile organisms, must continuously allocate limited resources between growth, development, reproduction, and defense against biotic and abiotic stress. This resource allocation dilemma underpins the concept of the growth-defense trade-off [1]. Because defense responses are energetically costly, their activation must be tightly coordinated with primary metabolism [2].

Plant defense against pests and pathogens relies on both constitutive barriers, such as physical structures and deterrent or antimicrobial metabolites, and inducible immune responses. In plant-herbivore interactions, immunity is triggered by herbivore-associated molecular patterns (HAMPs) and herbivore-derived effectors. Similarly, damage-associated molecular patterns (DAMPs), derived from endogenous plant molecules released upon injury or cell death, also contribute to immune activation. These signals induce rapid Ca^{2+} influxes and reactive oxygen species (ROS) bursts, which in turn activate mitogen-activated protein kinase (MAPK) cascades and trigger large-scale transcriptional reprogramming [3, 4]. Early signaling events converge on interconnected phytohormone networks centered on jasmonic acid (JA), salicylic acid (SA), and ethylene (ET). While SA predominantly mediates resistance to biotrophic pathogens, JA-ET signaling plays a central role in defense against necrotrophs and herbivorous attackers. Complex antagonistic and synergistic interactions between these pathways enable plants to fine-tune defense responses to specific threats [5].

Chloroplasts are central to this coordination between metabolism and immunity. In addition to their role in photosynthetic carbon assimilation, chloroplasts are key sites of JA, SA, and abscisic acid (ABA) biosynthesis and major regulators of cellular redox homeostasis. Perturbations in chloroplast electron transport during biotic stress initiate retrograde signaling to the nucleus, reinforcing defense gene expression. Plastids also modulate Ca^{2+} dynamics and ROS waves and influence the accumulation of defense-related metabolites, thereby shaping systemic immunity and priming [6–8]. Consistently, mutations in plastid-localized enzymes often alter hormone homeostasis and plant-herbivore interactions, positioning plastids as metabolic and signaling hubs integrating growth and defense [9–11].

Despite advances in understanding plant immunity, the genetic architecture underlying resistance to herbivorous chelicerates remains poorly understood. The two-spotted spider mite (*Tetranychus urticae*, TSSM) is one of the most destructive generalist herbivores worldwide and an increasingly important agricultural pest [12]. Natural accessions of *Arabidopsis thaliana* display extensive, polygenic variation in susceptibility to this mite. Although transcriptomic analyses indicate that *A. thaliana* responses to mite feeding resemble those triggered by other herbivores, highlighting JA signaling and indole glucosinolate biosynthesis as core defense components, these pathways do not fully explain the observed natural variation in susceptibility [13].

We therefore hypothesized that additional metabolic regulators, particularly those linking primary carbon metabolism with defense signaling, contribute to *A. thaliana* susceptibility to TSSM. To test this hypothesis, we applied genome-wide association study (GWAS) to identify loci associated with variation in mite susceptibility across natural accessions. Our results point to sugar signaling, and specifically the plastid-associated invertase *AtINVE*, as a potential modulator of plant-mite interactions. We propose that *AtINVE*-mediated sugar partitioning influences the growth-defense balance, thereby fine-tuning susceptibility to herbivore attack.

Materials and methods

Plant material

Seeds of *A. thaliana* L. accessions used in this study were obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK). The collection included 134 natural accessions (Table S1-3), a mutant line carrying a T-DNA insertion in the locus AT5G22510 (SALK_138953, *inve-1*, NASC ID: 670195), and the corresponding wild-type (WT) accession Col-8 (NASC ID: 60000).

All accessions were propagated for one generation under uniform growth conditions prior to experimentation. Seeds were stratified for 7 days at 4°C and subsequently transferred to growth chambers set to a 16 h/8 h day/night photoperiod, a light intensity of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 70% relative humidity, and a temperature of 22°C. For accessions requiring vernalization, 5-week-old plants were transferred to 4°C for 7–10 weeks under the same light regime and then returned to the conditions described above. Seeds collected after propagation were stored at 4°C.

Plants used in the experiments were sown on peat pellets (Jiffy, Netherlands) and grown for three weeks under the controlled conditions described above.

TSSM age-synchronized colony

An age-synchronized population of TSSM was obtained according to Złotkowska et al. [14]. Briefly, young females from a laboratory stock colony maintained on common bean (*Phaseolus vulgaris* L. cv. Ferrari; PNOS, Ożarów Mazowiecki, Poland) for more than 150 generations under controlled conditions were transferred onto detached leaves of two-week-old bean plants (50 females per leaf). The leaves were placed in Petri dishes lined with two layers of moist absorbent cellulose (Matopat, Toruń, Poland) and incubated in a growth chamber for 24 h under the same conditions used for *A. thaliana* growth.

After oviposition, the females were removed, and larvae hatched after approximately 4 days. Each leaf was then transferred to two-week-old bean plants and maintained in the same growth chamber to allow synchronous development of the mites to adulthood (approximately 2 weeks). Adult TSSM females at approximately five days post-emergence were used for all experiments.

A. thaliana plants infestation with TSSM

Ten five-day-old adult female mites originating from the age-synchronized colony were transferred to each experimental plant. Plants were maintained in a growth chamber under the same controlled conditions described above. For plants susceptibility measurements after 72 h of infestation, leaves were imaged using a Leica Thunder Imager system (Leica Microsystems Ltd., Switzerland) in reflected light at 25× magnification. For RNA isolation, enzymatic assays and soluble sugar analysis leaves were collected at the sixth hour of the light period and immediately frozen in liquid nitrogen before grinding.

Measuring A. thaliana susceptibility to TSSM

Susceptibility of *A. thaliana* plants to TSSM was assessed according to our previously described method [14]. High-resolution images were analyzed using the MITESPOTTER software to quantify damaged area, feces area, and feces number. Measurements were performed using eight biological replicates per

accession. Due to limited imaging throughput, biological replicates were randomized and processed in batches. Each batch included a reference accession (Col-0), allowing correction for potential variation between experimental runs. Final results were expressed as mean damaged area (mm²), feces area (mm²), and feces number, per plant per female for each accession and exported as comma-separated value (CSV) files.

GWAS analysis

The quantified phenotypic data were analyzed using the GWA-Portal platform [15]. Single nucleotide polymorphism (SNP) variants were obtained from the Imputed Full-Sequence Dataset (TAIR9) and from whole-genome resequencing data for diverse *A. thaliana* accessions [16–18]. GWAS was performed using the Accelerated Mixed Model (AMM) method to account for population structure and relatedness among accessions [19–21]. Only SNPs with a minor allele count (MAC) ≥ 10 were included in the analysis. Significance thresholds were determined using the Bonferroni correction based on the total number of SNPs tested. Manhattan plots were generated using the GWA-Portal platform.

RNA isolation and RT-qPCR analysis

Total RNA was isolated from three-week-old rosette leaves using the GeneMATRIX Universal RNA Purification Kit (EURx, Poland) according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Germany) following the manufacturer's protocol.

RT-qPCR was performed using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) with SG qPCR Master Mix (EURx, Poland) and primers listed in Table S6. Primer specificity was confirmed by agarose gel electrophoresis of PCR products. Relative gene expression was calculated using *AtAct2* (AT3G18780) and *AtPP2A* (AT1G69960) as reference genes. Reactions were performed in technical triplicate for each of three biological replicates. Melting curve analysis was performed after amplification to confirm the specificity of the PCR products.

Enzymatic assays

Enzyme extraction was performed according to Zhu et al. [22] with minor modifications. Briefly, 100 mg of ground leaf tissue was homogenized in 1 mL of ice-cold extraction buffer containing 50 mM HEPES-NaOH (pH 7.5), 1 mM EDTA, 5 mM Mg²⁺, 0.05% (v/v) Triton X-100, 1% (w/v) PVP-40, 1 mM DTT, and 1 mM PMSF. The homogenate was vortexed and incubated for 10 min at 4°C, followed by centrifugation at 20,000 × g for 10 min. The resulting supernatant was used for alkaline/neutral invertase (A/N-Inv) and vacuolar invertase (V-Inv) activity assays.

The pellet was washed three times with extraction buffer, resuspended in 100 μL of 1 M NaCl and 400 μL of extraction buffer, and incubated overnight at 4°C. After centrifugation at 20,000 $\times$ g for 10 min, the supernatant was used for the cell wall invertase (CW-Inv) activity assay.

Enzyme activity was measured in 100 μL reaction mixtures containing 30 μL enzyme extract and 70 μL reaction buffer supplemented with 100 mM sucrose. The reaction buffer consisted of 50 mM HEPES (pH 7.5) for A/N-Inv and 100 mM sodium acetate (pH 5.0 or 4.5) for V-Inv and CW-Inv, respectively. Reactions were incubated at 37°C for 1 h and terminated by adding 900 μL of 3,5-dinitrosalicylic acid (DNS) reagent prepared according to the standard protocol. Samples were then incubated at 95°C for 5 min. For V-Inv activity, the reaction mixture was diluted 1:1 with water (50 μL reaction mixture + 50 μL water) prior to DNS addition.

Blank controls contained 30 μL heat-inactivated enzyme extract and 70 μL of the corresponding reaction buffer. Reducing sugars released during the reaction were quantified spectrophotometrically by measuring absorbance at 540 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, USA). Enzyme activities were normalized to protein concentration determined using the RC DC Protein Assay Kit II (Bio-Rad, USA).

Soluble sugar analysis

Soluble sugars were extracted from leaf tissue according to Leach and Braun [23] with minor modifications. To 100 mg of frozen, ground tissue, 1 mL of ice-cold MCW extraction buffer (methanol:chloroform:water, 12:5:3, v/v/v) was added. Samples were vortexed thoroughly, incubated at 50°C for 30 min, and centrifuged at 14,000 rpm for 5 min at room temperature. The supernatant was transferred to a 15-mL conical tube and stored at 4°C. The extraction was repeated twice, and supernatants from the same sample were combined.

To separate phases, 0.6 volumes of water were added to the combined extracts, followed by vortexing and centrifugation at 4,700 $\times$ g for 5 min at room temperature. The volume of the aqueous (upper) phase was recorded. 3 mL of the aqueous phase containing soluble sugars was transferred into two 1.5-mL microcentrifuge tubes and dried under a nitrogen stream using the Reacti-Therm Heating and Stirring Module III TS-18823 (Thermo Fisher Scientific, USA). Samples were resuspended in 100 μL of water, and sucrose, glucose, and fructose contents were quantified using the Sucrose/D-Fructose/D-Glucose Assay Kit (Megazyme, Ireland).

Sugars were quantified spectrophotometrically by measuring absorbance at 340 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, USA).

Statistical analysis

Statistical analyses were performed in R [24] using RStudio [25]. Normality of residuals was assessed using the Shapiro-Wilk test. Student's *t*-test was used to compare two groups (wild type vs. *inve-1*) for susceptibility parameters. Invertase activity and soluble sugar content were analyzed using one-way ANOVA followed by Tukey's HSD test. Differences were considered significant at $p < 0.05$. Data are presented as mean $\pm$ standard error (SE), unless otherwise stated.

Data visualization was performed in R [24] using ggplot2 [26], ggdist [27], dplyr [28], and scales [29].

Results

TSSM susceptibility variation in the *A. thaliana* population

In this study, 134 *A. thaliana* accessions were analyzed, of which six were classified as relicts, while the remaining accessions (non-relicts) were assigned to nine distinct genetic groups (Fig. S1) [21].

Susceptibility to TSSM was determined based on three traits associated with mite infestation: damaged area, feces area, and feces number. These traits were automatically quantified using the MITESPOTTER program [14]. All three quantitative traits significantly differentiated the accessions within the population (Tables S1-3).

Relict accessions generally showed lower susceptibility to TSSM than non-relict accessions (Fig. 1a, c, e). The Can-0 accession from the Canary Islands exhibited higher susceptibility than the other relicts and was the most susceptible accession in terms of feces area and the third most susceptible based on damaged area (Table S1-2). Susceptibility varied widely across the population, with more than 18-fold, 6-fold, and 5-fold differences between the most extreme accessions based on damaged area, feces area, and feces number, respectively (Fig. S2).

The least susceptible accession based on damaged area was Car-1 (2.1 mm²), whereas VårA1 exhibited the lowest feces area (0.055 mm²) and feces number (45). The most susceptible accession based on damaged area was Stp-0 (38.82 mm²), while Can-0 showed the highest feces area (0.39 mm²) and Ale-Stenar-44-4 the highest feces number (235) (Tables S1-S3).

All three traits were strongly and positively correlated (Fig. 1b, d, f). Damaged area was more strongly correlated with feces area ($p = 6.18 \times 10^{-23}$, $r = 0.72$) than with feces number ($p = 7.47 \times 10^{-17}$, $r = 0.64$). The strongest association was observed between feces area and feces number ($p = 7.13 \times 10^{-32}$, $r = 0.80$).

Genome-wide association analysis of TSSM susceptibility

To identify genetic polymorphisms underlying the observed variation in TSSM susceptibility, we performed GWAS analysis (Fig. 2).

Residuals of damaged area and feces area values deviated significantly from normality, as assessed by the Shapiro-Wilk test ($p = 5.15 \times 10^{-8}$ and $p = 0.0099$, respectively), whereas residuals of feces number did not significantly differ from a normal distribution ($p = 0.1942$). Therefore, a Box-Cox transformation was applied, resulting in normally distributed trait values ($p = 0.24$ for damaged area and $p = 0.13$ for feces area). However, GWAS performed on transformed values for damaged area and feces area did not identify any statistically significant SNP associations. In contrast, analysis of untransformed damaged area values revealed a cluster of twelve SNPs on chromosome 5 exceeding a $-\log_{10}(p\text{-value})$ threshold of 8 and having a $\text{MAC} \geq 10$ (Fig. 2a; Table S4). At these loci, the minor allele was predominantly present in highly

susceptible accessions, with the four most susceptible accessions (Stp-0, Tamm-2, Can-0, and Pal-0) carrying all significant polymorphisms (Table S5).

No significant associations were detected for feces area or feces number, regardless of the data transformation method applied (none, logarithmic, square-root, or Box-Cox).

The associated SNPs spanned a genomic region between positions 7,413,142 and 7,476,817 on chromosome 5 of the reference *A. thaliana* genome. Within this region, including ± 20 kb flanking regions around the peak SNPs, 13 candidate genes potentially linked to plant susceptibility to TSSM were identified (Fig. 2b; Table 1).

Notably, two SNPs were located in intergenic regions between *AtFAR7* and *AtMWD9.23*. One SNP was located in the 5' UTR of *AtSCS1*, whereas eight SNPs were associated with *AtMWD9.26*, including one located in the 5' UTR or in an intron, depending on the splice variant, and seven located within an intron. One additional SNP was identified in the 5' UTR of *AtZPR1* (Fig. 2b).

Identification of candidate genes

Seven of the 13 candidate genes located within the genomic region harboring TSSM susceptibility-associated polymorphisms were selected for further analysis based on their predicted expression in leaf tissue. Among these, five genes are predicted to encode chloroplast-localized proteins, whereas two encode either a protein of the cytosolic large ribosomal subunit or a nucleus-localized protein. Of these seven genes, only four have characterized biological functions: *AtROPGAP1*, *AtRPL10AC*, *AtFAR1*, and *AtINVE* (Table 1).

We next examined the expression of these genes in leaves of the least susceptible (Car-1) and the most susceptible (Stp-0) accessions identified in our phenotyping screen based on damaged area (Fig. S2a, S3). This analysis revealed significant differences in basal gene expression between the two accessions, as well as distinct transcriptional responses following TSSM infestation (Fig. 3a-d).

AtROPGAP1 expression was generally higher in Stp-0 than in Car-1. In Stp-0, TSSM infestation caused a slight increase in expression, whereas in Car-1 transcript levels remained low and relatively stable, showing no significant response to TSSM attack (Fig. 3a).

Similarly, *AtRPL10AC* expression was slightly lower in Car-1 than in Stp-0 and decreased further following infestation in Car-1 plants (Fig. 3b).

AtFAR1 expression remained consistently low in Car-1 regardless of infestation status, whereas in Stp-0 expression levels were higher and decreased slightly following infestation (Fig. 3c).

AtINVE expression was higher in Car-1 than in Stp-0 under control conditions. In both accessions, TSSM infestation resulted in decreased expression (Fig. 3d).

Table 1
Candidate genes associated with plant susceptibility to TSSM

Locus name	Gene name	Encoded protein	Biological process	Location	Expression in leaves
AT5G22390	<i>AtMWD9.19</i>	FANTASTIC four-like protein	unknown	nucelus, cytosol	unknown
AT5G22400	<i>AtROPGAP1</i>	Rho GTPase-activity protein 1	signal transduction	chloroplast	+
AT5G22410	<i>AtRHS18</i>	Root hair specific 18	response to oxidative stress	extracellular region	-
AT5G22420	<i>AtFAR7</i>	Fatty acid reductase 7	suberin biosynthetic process	nucleus	-
AT5G22430	<i>AtMWD9.23</i>	Pollen Ole e 1 allergen and extensin family protein	unknown	membrane	-
AT5G22440	<i>AtRPL10AC</i>	Large ribosomal subunit protein UL1X	maturation of LU-rRNA, translation	cytosolic large ribosomal subunit	+
AT5G22450	<i>AtSCS1</i>	SAGA complex subunit	unknown	chloroplast	+
AT5G22460	<i>AtMWD9.26</i>	Alpha/beta-Hydrolases superfamily protein	unknown	cell wall, chloroplast, vacuole, plasmodesma	+
AT5G22470	<i>AtPARP3</i>	Poly(ADP-ribose) polymerase 3	double-strand break repair, protein poly-ADP-ribosylation	intracellular, nucleus	-
AT5G22480	<i>AtZPR1</i>	Zinc-finger domain protein	unknown	nucleus	+
AT5G22490	<i>AtMQJ16.3</i>	Wax ester synthase/diacylglycerol acyltransferase 9	triglyceride biosynthesis process	nucleus	unknown
AT5G22500	<i>AtFAR1</i>	Fatty acid reductase 1	response to salt stress, response to wounding, suberin biosynthesis process	chloroplast, plasma membrane	+

Locus name	Gene name	Encoded protein	Biological process	Location	Expression in leaves
AT5G22510	AtINVE	Alkaline/neutral invertase E	cotyledon development, starch metabolic process, sucrose catabolic process	chloroplast	+

Gene expression in leaf tissues and predicted protein subcellular localization were obtained from the Bio-Analytic Resource for Plant Biology (BAR) database (University of Toronto). Genes highlighted in grey met the following criteria: expression in leaf tissue and a characterized biological function. The gene selected for further analysis is indicated in bold.

Role of AtINVE during TSSM attack

Zhurov et al. [13] examined transcriptomic responses of susceptible and resistant *A. thaliana* accessions to TSSM infestation. Among differentially expressed genes (DEGs) were genes involved in sugar metabolism and transport, including invertases (*AtInvC* (AT3G06500), *AtCW-Inv1* (AT3G13790), *AtV-Inv1* (AT1G62660), *AtV-Inv2* (AT1G12240)), sucrose phosphate synthase (*AtSPS4F* (AT4G10120)), and sugar transporters (*AtSWEET15* (AT5G13170), *AtSTP13* (AT5G26340), *AtESL1* (AT1G08920), *AtERD6* (AT1G08930), *AtERDL6* (AT1G75220)). These findings suggest an important role for carbohydrate metabolism in plant-mite interactions. Moreover, sugar metabolism and allocation have repeatedly been identified as key factors shaping plant-herbivore interactions [30]. Based on these observations, we selected *AtINVE* for further analysis.

To investigate its role in TSSM susceptibility, we analyzed the *inve-1* knockdown mutant using damaged area, feces area, feces number, and oviposition rate as quantitative traits describing plant susceptibility.

The *inve-1* mutant plants were consistently more susceptible than WT plants across all measured traits (Fig. 4). The increase in feces area was statistically significant ($p = 0.026$), whereas increases in damaged area ($p = 0.06$) and feces number ($p = 0.055$) were close to statistical significance.

The A/N-InvE protein encoded by *AtINVE* catalyzes the hydrolysis of sucrose into glucose and fructose [31]. Consistent with this function, sucrose levels were slightly higher in *inve-1* than in WT plants prior to infestation, whereas glucose and fructose levels were slightly lower. Following TSSM infestation, sucrose and glucose levels decreased in both genotypes. In contrast, fructose levels in WT plants decreased to the level observed in *inve-1*, which remained largely unchanged before and after infestation (Fig. 5b, d, f).

A/N-Inv activity was slightly lower in *inve-1* than in WT plants and was not affected by TSSM infestation (Fig. 5a). V-Inv activity increased more than twofold in both WT and *inve-1* plants following infestation, with the mutant showing slightly higher activity than WT both before and after infestation (Fig. 5c). CW-Inv

activity also increased after infestation; however, these changes were not statistically significant between genotypes or treatments (Fig. 5e).

Given these differences between *inve-1* and WT plants, we further examined the most and least susceptible accessions to determine whether soluble sugar content and invertase activity are associated with TSSM susceptibility (Fig. 6).

The content of all three measured sugars decreased following TSSM attack. Sucrose and glucose levels were similar between the two accessions both before and after infestation (Fig. 6b,d). Before infestation, glucose content in Car-1 was slightly higher than in Stp-0. After TSSM attack, glucose levels in Car-1 decreased, resulting in lower glucose content than in Stp-0 (Fig. 6d). Prior to infestation, fructose content was slightly higher in Stp-0 than in Car-1. Following infestation, fructose levels in Stp-0 decreased to levels comparable to those observed in Car-1 before infestation (Fig. 6f).

The activity of all three invertase groups increased following TSSM infestation. A/N-Inv activity was similar between the two accessions before infestation and remained comparable afterward (Fig. 6a). V-Inv activity increased in both accessions, with the susceptible accession Stp-0 showing an increase of more than 1.5-fold (Fig. 6c). CW-Inv activity also increased by more than 1.5-fold in both accessions following infestation (Fig. 6e).

Discussion

From phenotypes to genes: unraveling susceptibility to TSSM in *A. thaliana* accessions

Using our phenotyping approach [14], we assessed 134 *A. thaliana* accessions and observed substantial variation in damaged area and feces production (both area and number), highlighting pronounced natural differences in susceptibility to TSSM. Relict accessions were generally among the least susceptible (Fig. S1). However, Can-0, a relict accession from the Canary Islands, ranked among the most susceptible based on damaged area (Table S1) and exhibited the highest feces area (Table S2), potentially reflecting its prolonged geographic isolation.

Interestingly, the four most susceptible accessions based on damaged area, together with the less susceptible accession Usa-0, carried the full set of significant SNPs (Table S5). This suggests that susceptibility is shaped by additional genetic and regulatory factors beyond the identified loci. Beyond these extremes, accessions from southern Sweden and the Iberian Peninsula displayed similar median susceptibility but substantial within-group variation (Fig. S1), consistent with the extensive diversity reported in natural populations [21]. The western European group was the most susceptible overall, whereas the admixed group showed particularly high variability (Fig. S1), likely reflecting recombination of alleles from diverse geographic origins. Collectively, these patterns underscore the influence of population structure and evolutionary history on plant-herbivore interaction.

Although damaged area and feces production were strongly correlated (Fig. 1b,d,f), GWAS identified loci significantly associated only with damaged area (Fig. 2). This discrepancy may result from the relatively

short feeding period used in our assay. Leaf damage provides a direct proxy for feeding intensity, whereas feces accumulation may require longer exposure to capture biologically meaningful variation. In addition, TSSM feces consist of both black feces and guanine pellets [32], whereas our MITESPOTTER software [14] currently quantifies only black feces. Incorporating guanine pellets in future analyses may improve trait resolution and increase GWAS power to detect loci associated with mite performance. Extending the feeding period may further clarify accession-specific differences in mite performance.

Differential gene regulation underlies contrasting susceptibility to TSSM

Differences in susceptibility between Car-1 and Stp-0 were associated with distinct transcriptional profiles of candidate genes (Fig. 3). *AtROPGAP1*, *AtRPL10AC*, and *AtFAR1* showed higher basal expression in the susceptible accession Stp-0, suggesting that their expression may promote conditions favorable for mite feeding. Notably, *AtROPGAP1* was induced following infestation in Stp-0 but remained stable in Car-1, indicating genotype-specific regulation. Although *AtFAR1* responded similarly to infestation in both accessions, its lower basal expression in Car-1 may restrict processes beneficial to TSSM. Together, these genes define a transcriptional profile associated with susceptibility in Stp-0 (Fig. 3a-c).

In contrast, *AtINVE* exhibited higher basal expression in Car-1. Although its expression decreased following infestation in both accessions, it remained elevated in Car-1 (Fig. 3d), suggesting that TSSM may actively suppress its expression to facilitate feeding. Functional analysis further supports a defensive role for *AtINVE*, as the *inve-1* knockdown mutant displayed increased susceptibility (Fig. 4). These findings indicate that *AtINVE* contributes to resistance, likely through modulation of sucrose metabolism and hexose availability. Among the identified candidates, *AtINVE* therefore emerges as a potential link between primary metabolism and defense responses.

AtINVE and carbohydrate metabolism in TSSM susceptibility

Disruption of *AtINVE* resulted in a reduction in total A/N-Inv activity, although this decrease was not statistically significant (Fig. 5a). A similar trend was observed in the natural accessions, where lower *AtINVE* expression in Stp-0 (Fig. 3d) corresponded to slightly lower A/N-Inv activity (Fig. 6a). However, *AtINVE* expression alone did not determine total A/N-Inv activity, as both accessions showed similar responses to TSSM infestation, suggesting functional redundancy among A/N-Inv isoforms.

Consistent with this, V-Inv and CW-Inv activities increased following infestation in both accessions, with no major differences in overall invertase activity between genotypes (Fig. 6a,c,e). These observations indicate that susceptibility is not determined by overall invertase activity alone. Instead, invertases likely act as regulators of metabolic signaling and have been identified as central integrators of stress responses [33]. For example, aphid infestation in pea induces dynamic changes in sugar metabolism and transport, redirecting carbon toward secondary metabolism and flavonoid biosynthesis [34]. Similar mechanisms have been described in other plant-herbivore systems. In *Nicotiana attenuata*, induction of the CW-Inv inhibitor NaCWII following *Manduca sexta* feeding limits apoplastic sucrose hydrolysis and reduces larval

performance [35]. In tomato, herbivory suppresses genes involved in sugar metabolism and transport, thereby restricting carbohydrate availability at feeding sites [36].

In our system, sugar concentrations and total invertase activity were broadly similar between accessions, whereas V-Inv and CW-Inv activities increased after infestation (Fig. 6c,e). These coordinated responses likely reflect a general metabolic adjustment to herbivory. Thus, sugar metabolism appears to influence susceptibility indirectly through dynamic redistribution of carbon resources rather than through absolute sugar levels. The reduced fructose content observed in the *inve-1* mutant (Fig. 5f) further supports a role for *AtINVE* in fine-tuning sugar homeostasis during TSSM attack.

Chloroplasts at the crossroads of defense and susceptibility

Five of the seven candidate genes identified by GWAS encode proteins predicted to localize to chloroplasts (Table 1), highlighting this organelle as a central hub of defense-related signaling. Chloroplasts are key sites of biosynthesis for defense hormones such as JA and SA, both of which contribute to responses against TSSM [37, 38, 13, 39, 40].

Chloroplasts also participate in the synthesis of secondary metabolites, including terpenoids, carotenoids, and indole glucosinolates, which act as deterrents to herbivores [41, 6, 42]. In addition, they regulate energy balance and carbohydrate allocation, thereby linking metabolic status with leaf nutritional quality [43]. The chloroplast-localized A/N-InvE [44] exemplifies how sugar metabolism intersects with defense pathways by modulating stromal sugar pools, potentially affecting defense metabolite biosynthesis and retrograde signaling.

Chloroplasts also function as dynamic signaling centers during biotic stress. Chloroplast-derived ROS and metabolic signals contribute to defense responses [6, 8], while stromules may facilitate communication between plastids and the nucleus [45]. Notably, increased stromule formation has been observed near mite feeding sites [46], supporting a role for plastid-derived signals in TSSM responses.

Together, these findings suggest that variation in chloroplast-targeted proteins may influence susceptibility through modulation of metabolic and signaling pathways. Similar steady-state sugar levels between accessions may therefore mask differences in carbon allocation toward defense metabolites, ROS production, or retrograde signaling.

Ecological and evolutionary relevance

Genotype-specific transcriptional and metabolic profiles likely reflect evolutionary trade-offs between growth and defense. The resistant accession Car-1 may prioritize sugar-mediated basal defense responses, whereas the susceptible accession Stp-0 may allocate resources toward processes that inadvertently increase susceptibility. Such differences could influence TSSM population dynamics in natural plant communities.

From an evolutionary perspective, these contrasting strategies may represent local adaptations to herbivore pressure. Natural selection may therefore shape distinct balances between resource allocation and defense investment across different geographic regions.

Conclusions

Our findings reveal that susceptibility to TSSM in *A. thaliana* is a complex trait shaped by genetic architecture, metabolic regulation, and evolutionary context. Natural variation among accessions reflects population structure and geographic history, while GWAS and candidate gene analyses point to the involvement of multiple interacting loci.

Although bulk sugar levels and total A/N-Inv activity showed limited variation, dynamic regulation of carbohydrate metabolism and associated signaling pathways appears to play an important role in defense. Susceptibility therefore seems to arise from coordinated metabolic and regulatory processes rather than from the action of individual genes.

Overall, this study highlights the ecological and evolutionary significance of natural variation and demonstrates the value of integrating phenotypic, genetic, and metabolic approaches to better understand plant-herbivore interactions.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Author Contribution

A.W. performed mutant phenotyping, GWAS analysis, analysis of phenotyping results, candidate genes analysis, primer design and validation, PCR, measurement of sugar content and invertase activity, manuscript writing; A.W., E.Z., P.D., E.S. and A.B.B. performed ecotypes phenotyping. A.B.B. and M.F. revised the manuscript. M.F. designed hypothesis. All authors reviewed the manuscript.

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Data Availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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Figures

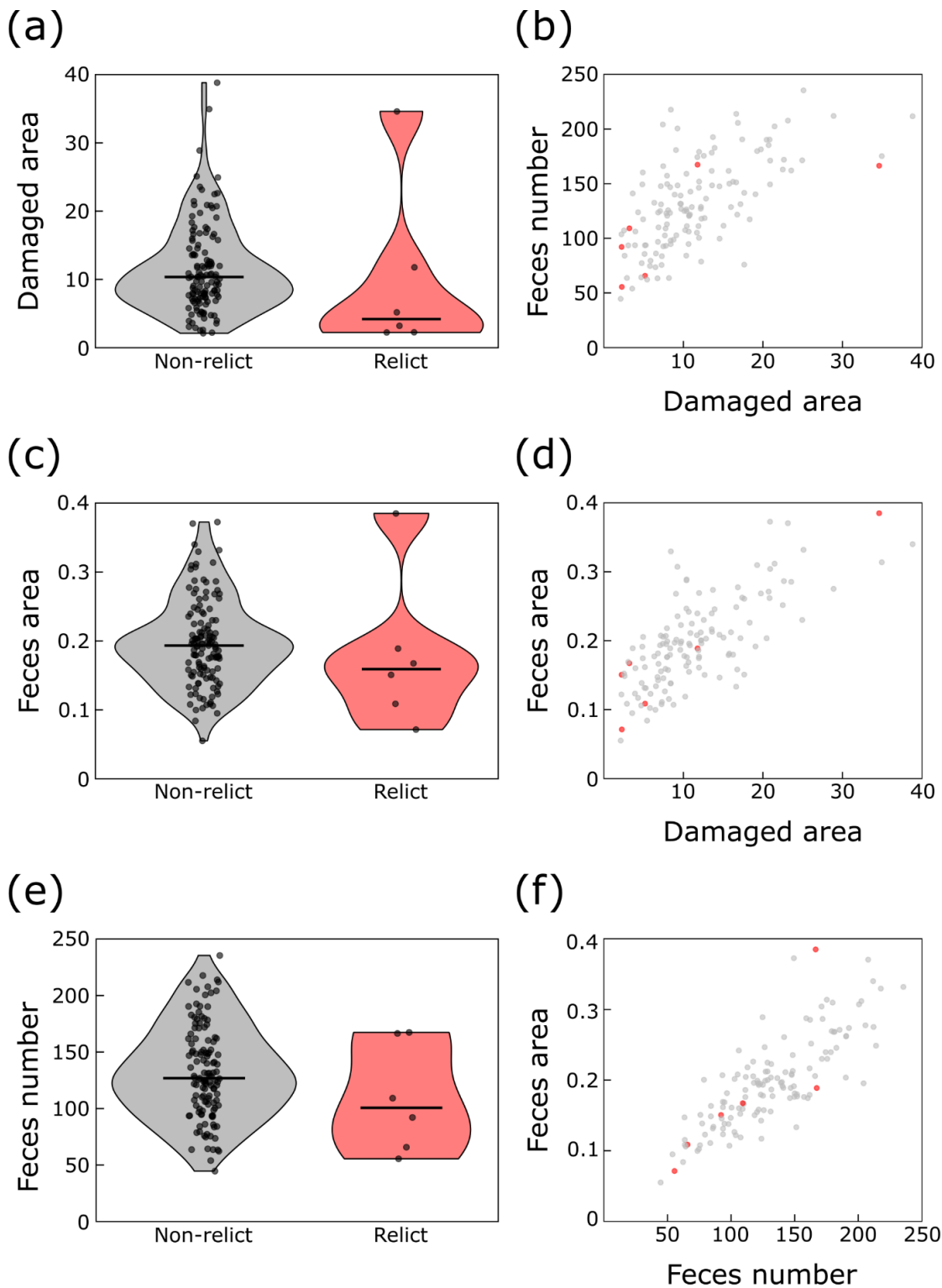


Figure 1

Trait differentiation and correlations between susceptibility-related traits in relict and non-relict *A. thaliana* accessions. Violin plots show the distribution of (a) damaged area (mm²), (c) feces area (mm²), and (e) feces number in non-relict (grey) and relict (red) accessions. Horizontal lines indicate median values. Scatter plots show correlations between susceptibility-related traits: (b) feces number and damaged area

(mm²), (d) feces area (mm²) and damaged area (mm²), and (f) feces area (mm²) and feces number. Points represent individual accessions.

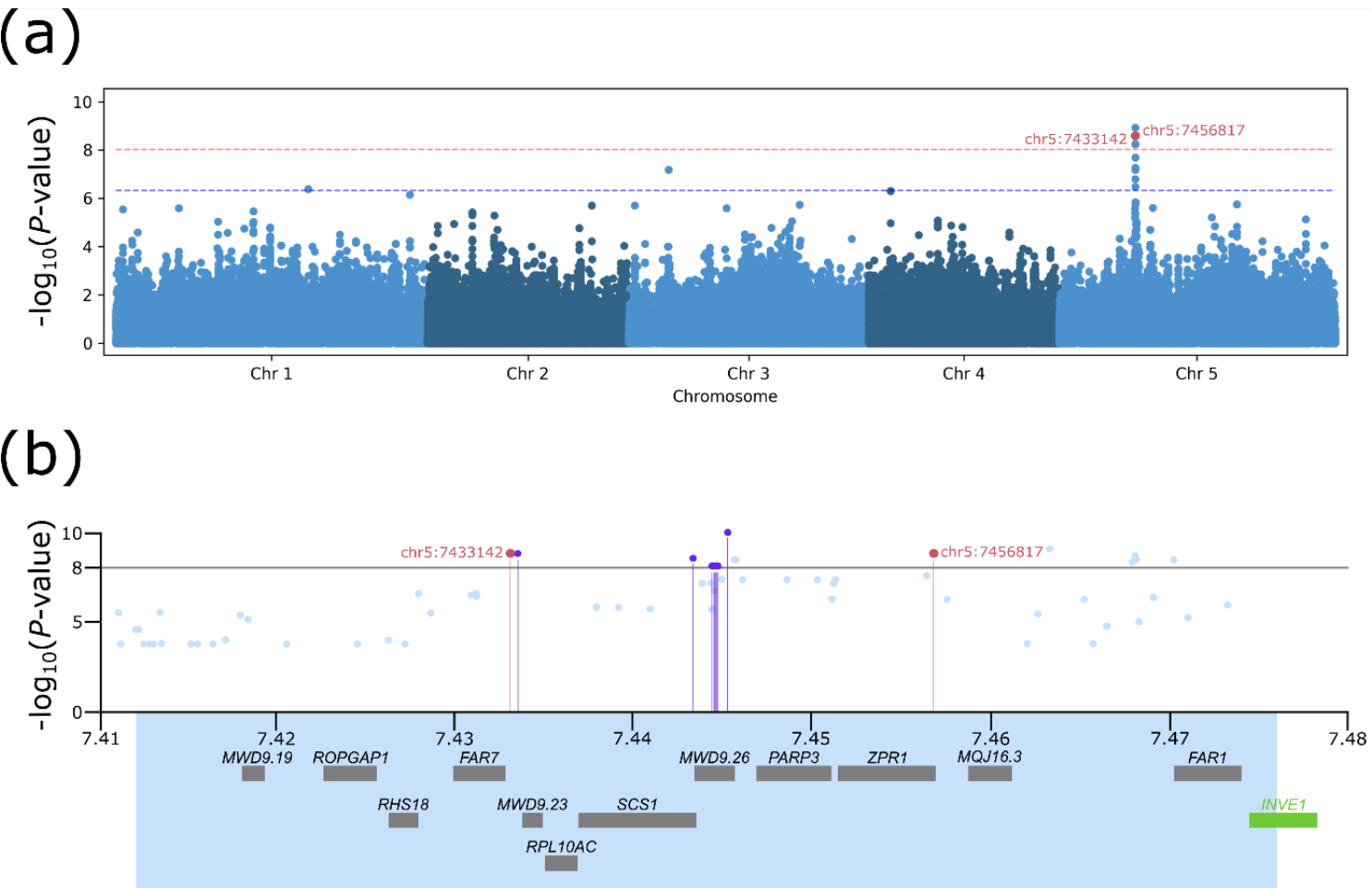


Figure 2

Genome-wide association analysis for damaged area. (a) Manhattan plot showing genome-wide single nucleotide polymorphism (SNP) associations with damaged area in 134 *Arabidopsis thaliana* accessions. Association significance is expressed as $-\log_{10}(p\text{-value})$ and was calculated using the Accelerated Mixed Model (AMM). Only SNPs with a minor allele count (MAC) ≥ 10 are shown. Alternating light and dark blue colors indicate individual chromosomes. (b) Enlarged view of the associated region on chromosome 5. All SNPs present in the studied population are shown in light blue together with gene annotations (Table 1). Flanking SNP positions (chr5:7,433,142 and chr5:7,456,817) are highlighted in red. Significantly associated SNPs ($-\log_{10}(p\text{-value}) \geq 8$; MAC ≥ 10) are shown in violet. The candidate gene *AtINVE*, selected for further analysis, is highlighted in green.

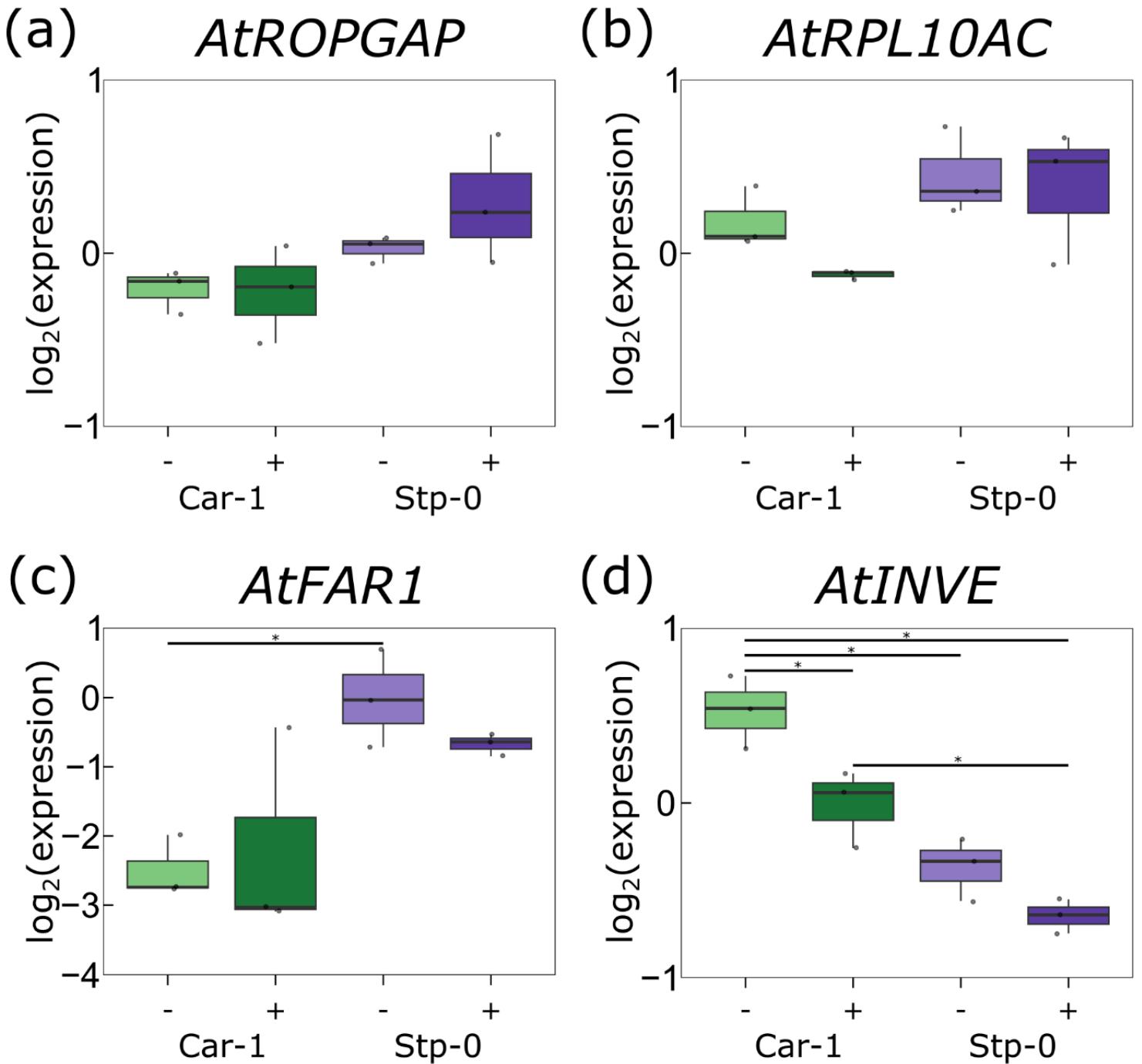


Figure 3

Relative expression of candidate genes in contrasting *Arabidopsis thaliana* accessions. Relative expression levels of (a) *AtROPGAP1*, (b) *AtRPL10AC*, (c) *AtFAR1*, and (d) *AtINVE* were measured in the least susceptible accession (Car-1; green) and the most susceptible accession (Stp-0; purple). Control (non-infested) conditions are indicated as (-), whereas plants infested with 10 adult female two-spotted spider mites per plant for 72 h are indicated as (+). Data represent means ± standard error (SE) from three biological replicates. Statistical significance was assessed using one-way ANOVA ($p < 0.05$); asterisks indicate significant differences between groups. Boxes represent interquartile ranges, horizontal lines indicate medians, and points represent individual measurements.

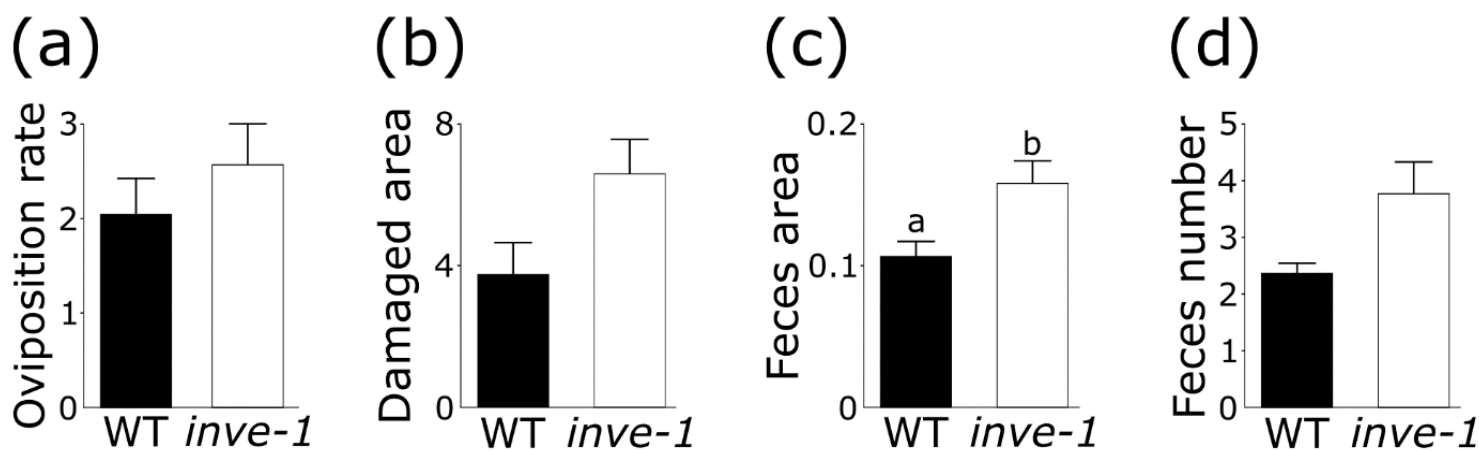


Figure 4

Susceptibility of the *Arabidopsis thaliana* *inve-1* knockdown mutant and wild-type plants to two-spotted spider mite. Susceptibility traits included (a) oviposition rate (number of eggs laid per female), (b) damaged area (mm² per female), (c) feces area (mm² per female), and (d) feces number per female. Measurements were performed in three-week-old rosettes of wild-type (WT) and *inve-1* plants infested with 10 adult female two-spotted spider mites per plant, shown as black and white bars, respectively. Data represent means $\pm$ standard error (SE) from three biological replicates. Statistical significance was evaluated using Student's *t*-test ($p < 0.05$). Different letters above the bars indicate statistically significant differences between groups.

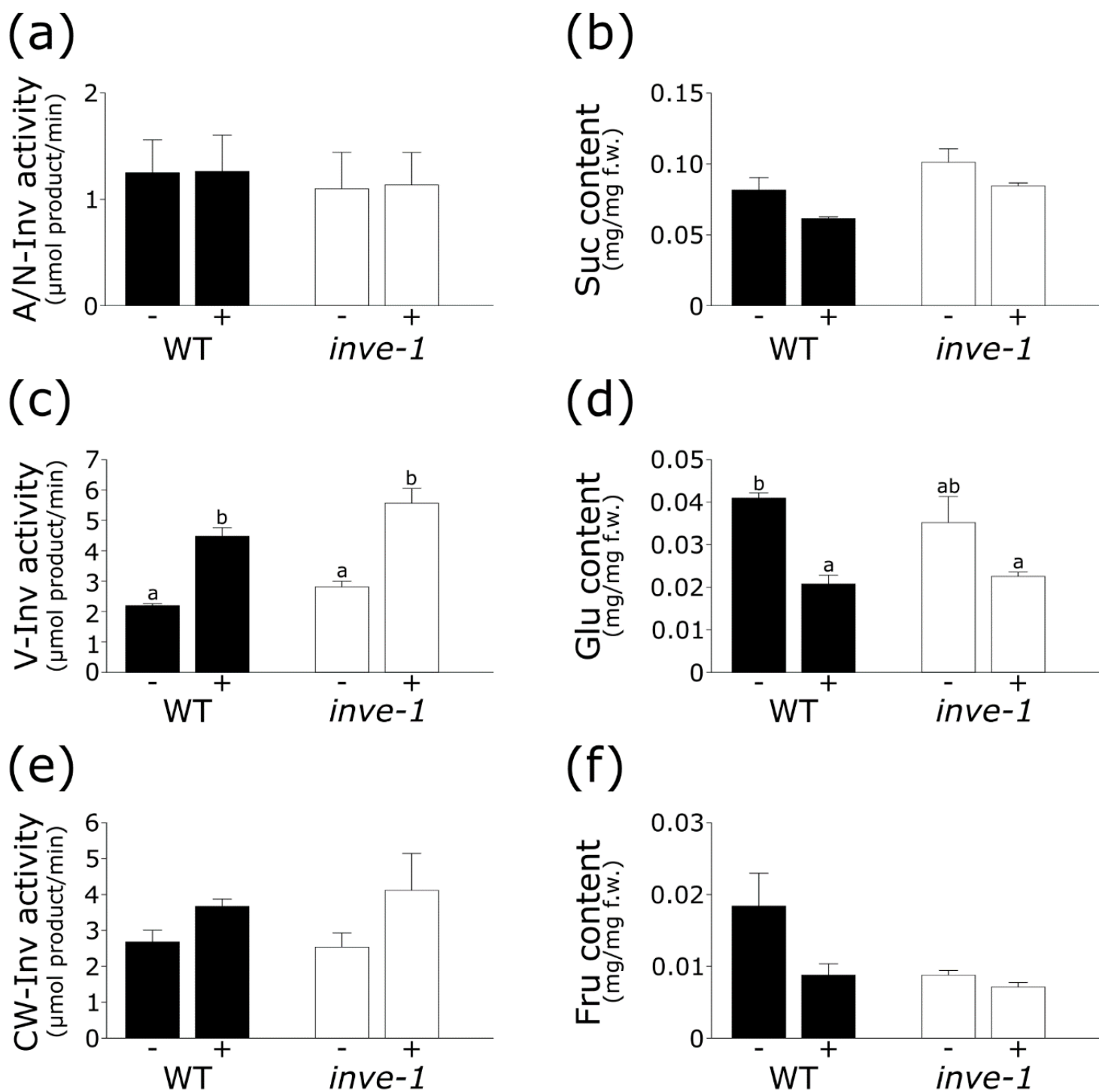


Figure 5

Invertase activity and soluble sugar content in the *Arabidopsis thaliana* *inve-1* knockdown mutant and wild-type plants. The activity of (a) alkaline/neutral invertases (A/N-Inv), (c) vacuolar invertases (V-Inv), and (e) cell wall invertases (CW-Inv) was measured and expressed as μmol of monosaccharides produced per minute. Soluble sugars were quantified as (b) sucrose (Suc), (d) glucose (Glu), and (f) fructose (Fru), and expressed as mg sugar per mg of rosette fresh weight. Measurements were performed on three-week-old rosettes of wild-type (WT) and *inve-1* plants, shown as black and white bars, respectively. Control (non-infested) conditions are indicated as (-), whereas plants infested with 10 adult female two-spotted spider

mites per plant for 72 h are indicated as (+). Data represent means $\pm$ standard error (SE) from three biological replicates. Statistical significance was assessed using one-way ANOVA ($p < 0.05$); different letters above the bars indicate statistically significant differences between groups.

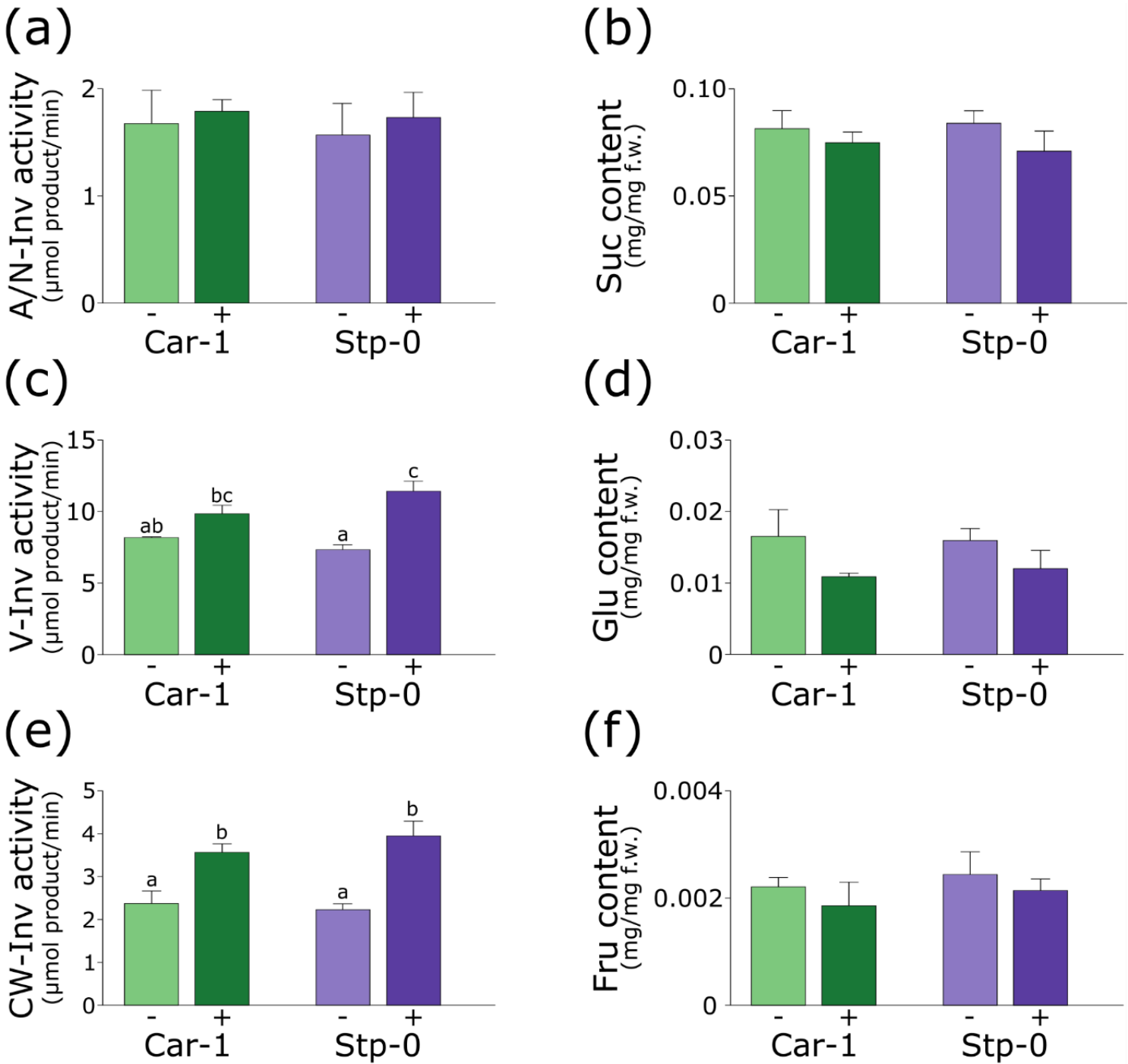


Figure 6

Invertase activity and soluble sugar content in contrasting *Arabidopsis thaliana* accessions. The activity of (a) alkaline/neutral invertases (A/N-Inv), (c) vacuolar invertases (V-Inv), and (e) cell wall invertases (CW-Inv) was measured and expressed as μmol of monosaccharides produced per minute. Soluble sugars were quantified as (b) sucrose (Suc), (d) glucose (Glu), and (f) fructose (Fru), and expressed as mg sugar per mg of rosette fresh weight. Measurements were performed on three-week-old rosettes of least susceptible

accession (Car-1; green) and the most susceptible accession (Stp-0; purple). Control (non-infested) conditions are indicated as (-), whereas plants infested with 10 adult female two-spotted spider mites per plant for 72 h are indicated as (+). Data represent means $\pm$ standard error (SE) from three biological replicates. Statistical significance was evaluated using one-way ANOVA ($p < 0.05$); different letters above the bars indicate statistically significant differences between groups.

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