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## Induced plant-defenses suppress herbivore reproduction but also constrain predation of their offspring

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### ABSTRACT

Inducible anti-herbivore defenses in plants are predominantly regulated by jasmonic acid (JA). On tomato plants, most genotypes of the herbivorous generalist spider mite *Tetranychus urticae* induce JA defenses and perform poorly on it, whereas the Solanaceae specialist *Tetranychus evansi*, who suppresses JA defenses, performs well on it. We asked to which extent these spider mites and the predatory mite *Phytoseiulus longipes* preying on these spider mites eggs are affected by induced JA-defenses. By artificially inducing the JA-response of the tomato JA-biosynthesis mutant *def-1* using exogenous JA and isoleucine (Ile), we first established the relationship between endogenous JA-Ile-levels and the reproductive performance of spider mites. For both mite species we observed that they produced more eggs when levels of JA-Ile were low. Subsequently, we allowed predatory mites to prey on spider mite-eggs derived from wild-type tomato plants, *def-1* and JA-Ile-treated *def-1* and observed that they preferred, and consumed more, eggs produced on tomato plants with weak JA defenses. However, predatory mite oviposition was similar across treatments. Our results show that induced JA-responses negatively affect spider mite performance, but positively affect the survival of their offspring by constraining egg-predation.

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### 1. Introduction

Plants are constantly attacked by herbivores and have evolved a rich palette of traits associated with resistance, such as mechanical barriers, toxins and feeding deterrents to defend themselves against these attackers [1]. Many of the defense responses that enable plants to resist herbivores are inducible probably because displaying them constitutively would be energetically more costly and may cause auto-toxicity [2]. Moreover, induced defenses allow for a certain degree of tailoring of resistance to specific attackers [3]. Constitutive and induced defenses can operate directly or indirectly. While the direct defenses are established via toxins, deterrents or structural barriers, the indirect defenses require

recruitment by the plant of an herbivore's natural enemy [2,4]. Together direct and indirect defenses make up the toolbox plants can use to combat herbivores, while the vulnerability of the herbivores to these defenses determine the extent to which plants can resist them. However, there is a potential conflict looming since direct defenses may not only decrease the performance of herbivores, but also of natural enemies that prey on these herbivores, and thereby ingest the same defensive substances [5–7].

Tomato plants are relatively hostile hosts for herbivores, where constitutive and inducible defenses have been documented in detail [8,9]. The inducible defensive system is orchestrated by several phytohormones of which jasmonic acid (JA) is a central player, given the fact that JA-biosynthesis or JA-perception mutants are highly vulnerable to herbivores and biotrophic pathogens [10,11]. For instance, the tomato JA biosynthesis-mutant *defenseless-1* (*def-1*) [10] is highly vulnerable to the larva of *Manduca sexta* [10] and *Spodoptera exigua* [12] and the spider mite *Tetranychus urticae*

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[13,14], while 35S:Prosystemin tomato, a transgenic plant primed to display exceptionally strong JA-defenses, is more resistant to herbivore attack [15]. In fact, not JA itself, but its isoleucine (Ile) conjugate, i.e. JA-Ile, binds to the receptors COI1 and JAZ protein and is the bioactive jasmonate responsible for the expression of defense genes [16,17] and concomitant downstream metabolic changes. Some of these defense genes are commonly used as JA-markers, i.e. as indicators of activated JA-defenses. In tomato, wound-induced proteinase inhibitors (PI), such as *PI-IIf*, are commonly used as JA-markers [13,14,18–21].

Tomato plants that may have a relatively high degree of disease and pest resistance yet suffer from several adapted pests and pathogens [22,23]. Among these pests are several species of herbivorous mites such the spider mites *Tetranychus urticae* and *Tetranychus evansi*. Performance of these mites on tomato is negatively correlated with PI transcript accumulation levels or enzyme activity [14,15,24] and with the presence of the terpene 7-epizingiberene [25] and methyl ketones [26]. In tomato PI-activity and 7-epizingiberene production can be induced by JA [13,14,27]. However, while these defenses may decrease the reproductive performance of maladapted spider mites significantly, they do often not prevent them from building a population [15]. In addition, spider mites were found to adapt to these defenses as well. Two distinct types of adaptations to tomato JA-defenses have been reported. The first type is resistance: although most populations of *T. urticae* initially perform poorly on tomato, they were found to often improve their reproductive performance when propagated on this plant for several generations [28,29]. The second type is suppression: some *T. urticae* genotypes can suppress inducible JA-defenses to intermediate levels [15,21], while *T. evansi* suppresses these defenses down to [21] or below [24], the levels of uninfested plants. The fact that such adaptations appear to be relatively common in natural populations of *T. urticae* and *T. evansi* [21] suggests that JA-defenses pose a constant selective pressure on these mites.

Under laboratory conditions, JA-sensitive mites were found to benefit from residing on the same leaf as JA-suppressing mites [15,21] implying that defense suppression may backfire within herbivore communities by facilitating competitors. This notion is reinforced by the observation that suppressing JA-defenses was only beneficial to JA-defense sensitive mites when these could monopolize their feeding site [21,30]. These observations warrant to further explore the circumstances under which defense suppression is or is not beneficial for herbivores. It is well known that ingested plant defense compounds sometimes protect herbivores against their natural enemies [5,6,31,32]. This suggests that inducing defenses may actually be beneficial to resistant herbivores, since it may constrain predation pressure, while suppressing defenses may promote it. If so, we would expect the inducer spider mite *T. urticae* to experience less predation pressure than its competitor *T. evansi* that suppresses defenses. However, the opposite has been observed: most of the predators used in biological control of spider mites, such as the phytoseiids *Phytoseiulus persimilis* [33,34], *Neoseiulus californicus* [34], *Phytoseiulus macropilis* [35] and *Phytoseiulus fragariae* [36] are not efficient in controlling *T. evansi*, although yet another species, *Phytoseiulus longipes*, may be suitable for this purpose [37,38]. The reasons why *T. evansi* seems unpalatable to most predators are not known [39]. In addition, suppression of defenses may also impair the attraction of natural enemies and result in enemy free space, although the predators *P. macropilis* and *P. longipes* were found to prefer the odors of *T. evansi*-infested plants over uninfested controls despite suppression of several induced plant volatiles [24]. Taken together, much is still unclear about the costs and benefits of defense suppression and how this trait emerges and persists in natural populations.

We asked to which extent suppression of JA-defenses by spider mite *T. evansi* would affect performance of the mite itself, as well

as predation by the predatory mite *P. longipes*. First, we manually induced the JA-response of the tomato JA-biosynthesis mutant *def-1* using exogenous JA + isoleucine (Ile) and used this assay to assess the relationship between *T. evansi* performance and the magnitude of the JA-defenses. Then, we tested to which extent these defenses modulate egg predation, since *P. longipes* rarely attacks adult spider mites and prefer to eat their eggs [40], in terms of prey-egg preference, prey-egg consumption rate and predatory mite oviposition. These same tests we performed using the eggs of a JA-defense inducer strain of *T. urticae* [15,21,30], as a benchmark for the impact of induction.

## 2. Materials and methods

### 2.1. Plants

Bean (*Phaseolus vulgaris* cv. Speedy), wild type tomato *Solanum lycopersicum* cv. Castlemart and mutant *def-1* (also in the cv. Castlemart genetic background [10]) plants were grown in a greenhouse with day/night temperatures of 18–25 °C, a 16:8 h (light:dark) regime and 50–60% relative humidity (RH). Cucumber plants (*Cucumis sativa* L. cv. Ventura) were grown in a climate room at 25 °C, 16:8 h (light:dark) and 60% RH (default settings). Bean and cucumber leaves were only used for propagating mites and tomato plants were used for experiments when they were 28 days old. Two days prior to the start of an experiment, all tomato plants were transferred to a climate room where the experiments were carried out.

### 2.2. Spider mites

The *T. urticae* strain used for this study was obtained from a natural population collected from spindle tree (*Euonymus europaeus*) in the Netherlands, referred to as “KMB” in Kant et al. [15] and as “Santpoort 2” in Alba et al. [21] in which it is described in detail. The *T. evansi* strain used for this study was originally collected from tomato plants in Brazil, referred to as *T. evansi* in Sarmento et al. [24] and as Viçosa-1 in Alba et al. [21] in which it and its interaction with tomato are described in detail. Both species were propagated on detached leaves (i.e. bean leaves for *T. urticae* and tomato leaves for *T. evansi*) laying flat on wet cotton wool inside plastic trays in a climate room. Fresh leaves were provided 3 times per week.

For all experiments we used adult female mites;  $3 \pm 1$  days since their final molt. To obtain them we transferred adult females of *T. urticae* or *T. evansi* to detached bean leaves on wet cotton wool. After 48 h these adult females were removed and their eggs were left to hatch and mature to adulthood in a climate room. Since eggs to adult take about 14 days, we transferred the females to the bean leaves 17 days before the start of the experiments.

### 2.3. Predatory mites

The base colony of the predatory mite *P. longipes* had been provided by Koppert Biological Systems (Berkel and Rodenrijs, the Netherlands) in 2011 and had been maintained in a climate room on detached cucumber leaves infested with *T. urticae*. For the experiments, cohorts of predatory mite eggs were obtained by placing adult females on detached cucumber leaves (placed flat on wet cotton wool) and allowing them to produce eggs for 24 h. Subsequently, the adults were removed and the eggs were allowed to mature on these leaves under the same conditions as the base colony. From this cohort, adult predatory mite females were used when they were 8–9 days old since egg stage, i. e.  $2 \pm 1$  days since their final molt.

#### 2.4. Treatment of *def-1* tomato leaflets with JA and Ile

Tomato *def-1* is unable to significantly accumulate wound-induced or herbivore-induced JA and JA-Ile [10,13]. This makes *def-1* an ideal mutant for studying induced JA defenses and to manipulate these manually. We restored JA-induced defenses in detached tomato *def-1* leaflets by placing their petiolules in a conical centrifuge tube (15 ml; Greiner) containing tap water with 0.05 mM ( $\pm$ )-jasmonic acid (JA) and 1 mM isoleucine (Ile) (Sigma-Aldrich); the extra Ile was added to make sure the leaflets could respond maximally to the JA added in the solution, as pointed by Bar Nun and Mayer [41]. The JA stock-solution was prepared as described in Ament et al. [14]. As controls we used leaflets placed in tap water or in tap water with 1 mM Ile. We detached 5 fully expanded leaflets with their petiolules per tomato plant and randomly assigned them to the different treatments. Once a leaflet had been placed with its petiolule in a tube, the remaining opening of the tube was gently plugged with a piece of wet cotton wool around the petiolule to fix the leaflet. Then, a thin line of insect glue (Biocontrole, São Paulo, Brazil) was applied around the opening of the tube to prevent mites from escaping. Tubes with detached leaflets were placed horizontally to make sure the petiolule would be in contact with the fluid during the course of the experiment.

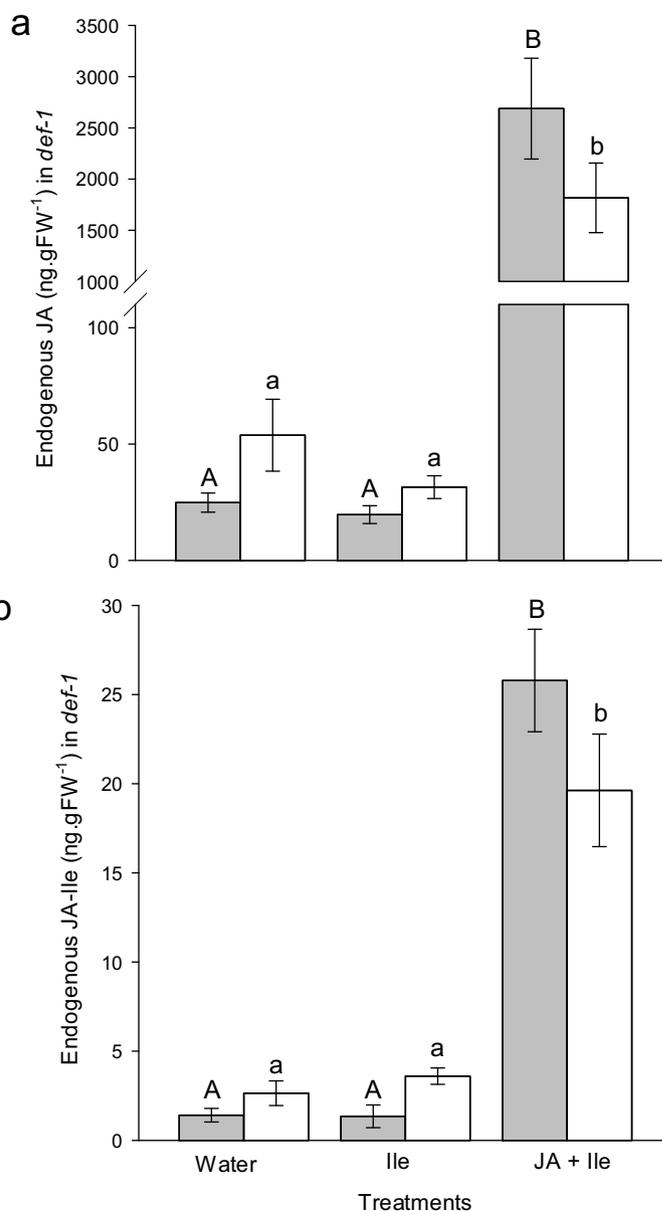
#### 2.5. Spider mite performance assay on *def-1* tomato leaflets treated with JA + Ile

Spider mite performance was measured by assessing peak oviposition, which has been indicated as a good proxy for the rate of population increase by Janssen and Sabelis [42] and was validated for *T. evansi* and *T. urticae* in Sarmento et al. [24]. Experiments were performed with *def-1* leaflets that had been exposed for 24 h to their treatment solution. To assess spider mite performance on *def-1* leaflets with an induced JA-related defense we used a soft bristle paintbrush to transfer 15 adult female spider mites to each leaflet. After 48 h, the number of eggs was assessed using a stereomicroscope. Directly after egg counting, the leaflets were removed from the tubes, the petiolules were cut off with a razor blade to prevent sample contamination with JA and Ile. Afterwards, the leaflets were flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  to determine their JA and JA-Ile content (as described in more detail further down in the methods section). This experiment was performed twice independently in time with 8–10 leaflets from 2 different plants per treatment (occasionally a leaflet dried out during the course of an experiment and was thus, excluded from the analysis).

##### 2.5.1. Statistical analysis

Differences in the JA or JA-Ile accumulation among the treatments were first analyzed using a linear mixed-effects model (LMER) using the lme4 package in R version 2.15.1 [43]. Since 5 leaflets were collected from the same tomato plant (*def-1*) and the experiments were carried out in two blocks in time, we built a LMER model including “plant” and “time” as random factors. Because these random factors did not have significant effects on the JA-Ile accumulation we decided to use the simplest model, i.e. a model without these random factors. Hence, the analysis was redone using a generalized linear model (GLM) with a normal error distribution, with JA or JA-Ile accumulation as response variable (*y*) and treatments (water, Ile, JA + Ile) as explanatory variable (*x*) [44]. JA and JA-Ile accumulation were log-transformed prior to the analysis and the data are presented in Fig. 1.

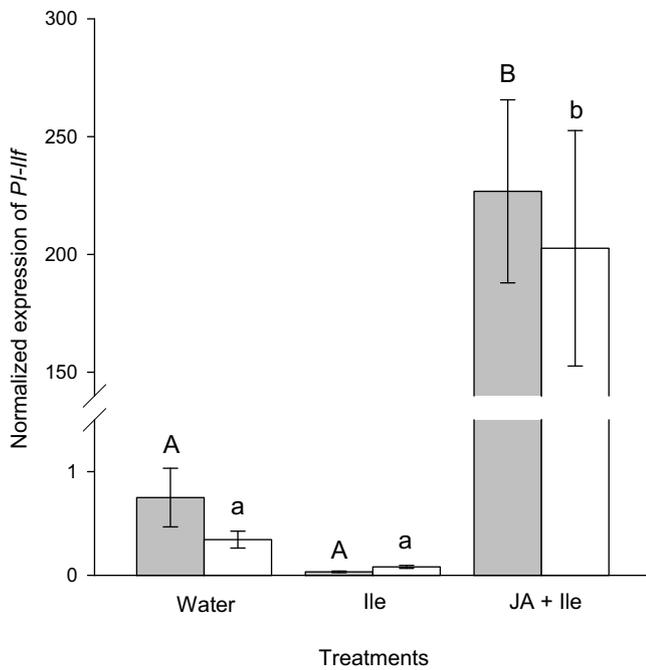
To assess differences in the reproductive performance of *T. evansi* and *T. urticae* on *def-1* leaflets treated with water, Ile or JA + Ile we calculated the average number of eggs laid per female per day (referred to as “oviposition rate” in the figures) on each treatment. First, we analyzed the data using LMER, in which “plant” and “time”



**Fig. 1.** JA and JA-Ile accumulation after 48 h in spider mite-infested tomato *def-1* leaflets. Leaflets of the JA-biosynthesis mutant *Solanum lycopersicum def-1* had been placed in tap water (“Water”), tap water + Ile (“Ile”) or tap water + JA + Ile (“JA + Ile”) for 24 h. Subsequently, 15 adult female spider mites were introduced and were allowed to feed for 48 h after which the leaflets were assayed for their endogenous JA and JA-Ile levels by means of LC-MS. Panel a shows the mean concentration ( $\text{ng g FW}^{-1}$ )  $\pm$  SE of JA and panel b) shows the mean concentration ( $\text{ng g FW}^{-1}$ )  $\pm$  SE of JA-Ile. Gray bars indicate the phytohormone levels in *T. evansi* infested leaflets. White bars indicate the phytohormone levels in *T. urticae* infested leaflets. Different uppercase letters indicate significant differences for *T. evansi* treatments and lowercase for *T. urticae* ( $P < 0.05$ ).

were included as random factors, but these did not have significant effects on the oviposition rate. Hence, the analysis was redone using GLM with a normal error distribution [44]. The data are presented in Fig. 4a.

We evaluated the relationship between the reproductive performance of the mites and the JA-Ile content of the leaflets on which they had fed using a non-linear regression model (nls) in R with a normal error distribution, with the oviposition rate as response variable (*y*) and JA-Ile as explanatory variable (*x*). The Akaike information criterion was used to select the model equation [44] most accurately explaining the variation in the oviposition



**Fig. 2.** *PI-Ilf* expression after 48 h in spider mite-infested tomato *def-1* leaflets. Leaflets of the JA-biosynthesis mutant *def-1* had been placed in tap water (“Water”), tap water + Ile (“Ile”) or tap water + JA + Ile (“JA + Ile”) for 24 h. Then 15 adult female spider mites were introduced and were allowed to feed for 48 h after which the *def-1* leaflets were assayed *PI-Ilf* transcript levels. Gray bars indicate the transcript levels in *T. urticae* infested leaflets. White bars indicate the expression levels in *T. urticae* infested leaflets. Different uppercase letters indicate significant differences for the *T. evansi* treatments and lower case for *T. urticae* ( $P < 0.05$ ). *PI-Ilf* transcript levels were assessed by means of RT-qPCR. Bars represent the mean normalized expression (NE)  $\pm$  SE.

of the spider mites and a non-linear curve was generated accordingly. For *T. evansi* the dose-response was described by the function  $y = a + (b / (1 + e^{(x)}))$  with  $a = 3.8 \pm 0.4$  (1 SE) and  $b = 3.5 \pm 1.7$  (1 SE,  $n = 25$ ) and for *T. urticae* by the function  $y = a + (b / (1 + e^{(c-x)}))$  with  $a = 5.9 \pm 0.3$  (1 SE);  $b = -2.5 \pm 0.5$  (1 SE) and  $c = 7.9 \pm 1.3$  (1 SE,  $n = 28$ ). The data are presented in Fig. 4b.

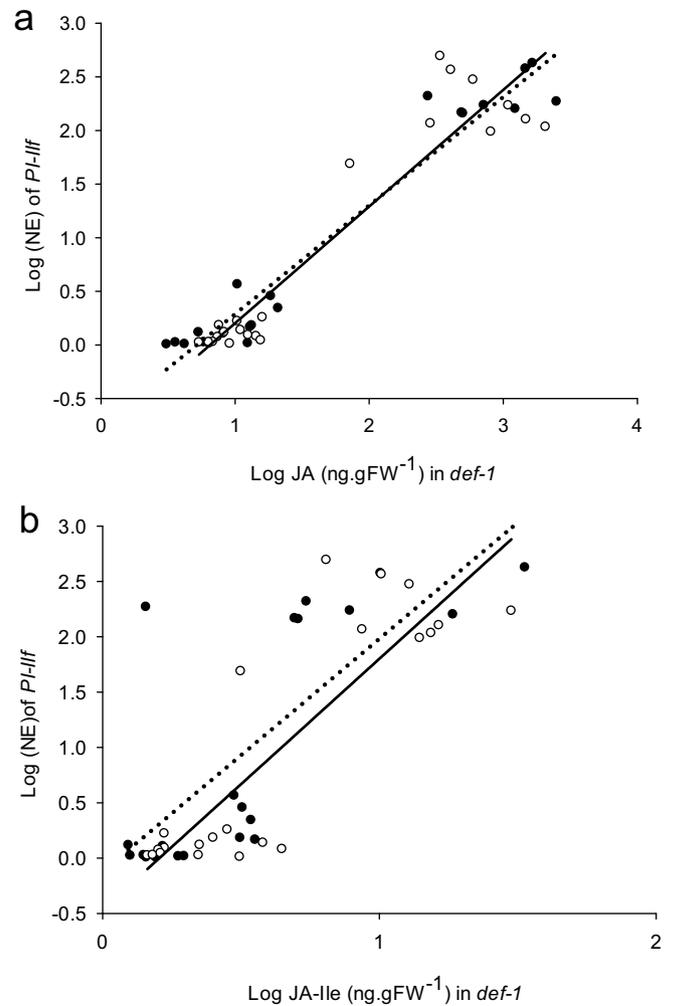
## 2.6. Phytohormone accumulation versus gene expression on JA + Ile treated *def-1* tomato leaflets

Experiments were performed following the same procedure described above, i. e. we exposed *def-1* leaflets for 24 h to their treatment solution, transferred 15 mites to each leaflet and waited for another 48 h before sampling the leaflets. Aiming to correlate phytohormone levels with gene expression, data of JA and JA-Ile accumulation and gene expression of the JA-marker gene *PI-Ilf* were obtained from the same samples. This experiment was performed twice independently in time with 8–10 leaflets (occasionally a leaflet dried out and was, therefore, excluded from the analysis).

### 2.6.1. Statistical analysis

First, we analyzed the data using LMER, in which “plant” and “time” were included as random factors, but these did not have significant effects on the JA or JA-Ile accumulation after model simplification. Hence, the analysis was redone using GLM with a normal error distribution, with normalized expression (NE; explained further down in the methods section) of *PI-Ilf* as response variable ( $y$ ) and treatments (water, Ile, JA + Ile) as explanatory variable ( $x$ ). The data are presented in Fig. 2.

The coefficient of determination ( $R^2$ ) was used to detect the best fit between dependent and independent variables. The highest  $R^2$  was obtained fitting the log transformed JA levels and the log



**Fig. 3.** The relationship between JA and JA-Ile accumulation levels with the expression of *PI-Ilf*. Leaflets of the JA-biosynthesis mutant *def-1* had been placed in tap water (“Water”), tap water + Ile (“Ile”) or tap water + JA + Ile (“JA + Ile”) for 24 h. Then, 15 adult *T. evansi* (filled circles, dotted line) or *T. urticae* (open circles, solid line) females were introduced and were allowed to feed for 48 h after which the *def-1* leaflets were assayed for endogenous JA and JA-Ile levels (Fig. 1) and *PI-Ilf* expression (Fig. 2). The regression plot a shows the relationship of log JA and log *PI-Ilf*; panel b shows the relationship of log JA-Ile and log *PI-Ilf* across all samples.

transformed NE of *PI-Ilf* into linear regression models. The same outcome we obtained for JA-Ile levels and NE of *PI-Ilf*. In more details, the regression analysis of JA and *PI-Ilf* was described by the function  $y = 1.0116x - 0.7215$  ( $R^2 = 0.95$ ) for *T. evansi* ( $n = 21$ ) and by the function  $y = 1.0877x - 0.8843$  ( $R^2 = 0.89$ ) for *T. urticae* ( $n = 22$ ). The data are presented in Fig. 3a. The regression analysis of JA-Ile and *PI-Ilf* for *T. evansi* ( $n = 21$ ) was described by the function  $y = 2.1032x - 0.1218$  ( $R^2 = 0.58$ ) and for *T. urticae* ( $n = 22$ ) by the function  $y = 2.2652x - 0.4639$  ( $R^2 = 0.74$ ). The data are presented in Fig. 3b.

## 2.7. Phytohormone extraction and analysis by means of LC-MS/MS

Phytohormone extraction and subsequent analysis by means of LC-MS/MS was performed using the procedure of Alba et al. [21]. In short, around 200 mg of frozen leaf material was weighed and homogenized in 1 ml ethyl acetate spiked with D<sub>5</sub>-JA (C/D/N Isotopes Inc., Canada) as internal standard at a final concentration of 100 ng ml<sup>-1</sup>. The pellet was re-extracted with 0.5 ml ethyl acetate without the internal standard and evaporated to dryness. The

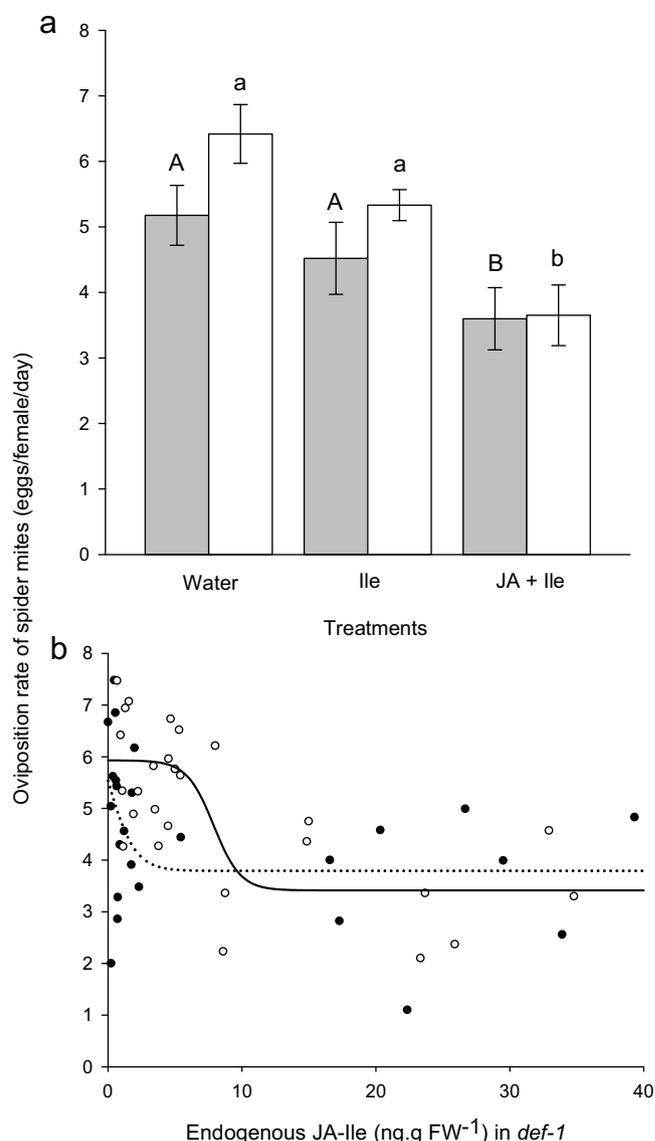
residue was re-dissolved in 500  $\mu$ l methanol 70% (v/v) and, then, analyzed by means of LC–MS/MS (Varian 320–MS triple quadrupole LC–MS/MS, Agilent Technologies, Santa Clara, California, US). A serial dilution of pure standards of JA and JA-Ile were run separately. JA and JA-Ile were quantified by comparing the sample peak area with the peak area of the respective external standard. The internal standard D<sub>5</sub>-JA was used to estimate the recovery rate of JA and JA-Ile and their endogenous concentrations were subsequently quantified using the external standard series. Phytohormone amounts were expressed as ng per gram of leaf fresh weight (ng gFW<sup>-1</sup>).

### 2.8. Gene expression analysis by means of RT-qPCR

Total RNA of each tomato leaflet was isolated with a phenol–LiCl-based method as described in [45]. The final total RNA concentration was assessed using the Nanodrop 1000 (Thermo Scientific). An amount of 4  $\mu$ g total RNA was treated with TurboDNase (Ambion) in a 20  $\mu$ l reaction, after which 10  $\mu$ l was used for cDNA synthesis using M-MuLV RT (Fermentas) according to the protocols of the manufacturers. The cDNA was 10x diluted and 1  $\mu$ l was subsequently used as template for qPCR using the Platinum SYBR Green qPCR-SuperMix-UDG kit (Invitrogen) and the ABI 7500 Real-Time PCR system (Applied Biosystems). Reactions were performed according to the instructions of the manufacturers in a volume of 20- $\mu$ l, containing 0.25  $\mu$ M of each primer, 0.1  $\mu$ l ROX reference dye and 1  $\mu$ l of cDNA template. The cycling program was set to 5 min of pre-cycling stage (50 °C), 5 min at 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 60 °C followed by a melting curve analysis. The amplicon identities were verified by Sanger sequencing. Normalized expression (NE) was calculated by the  $\Delta$ Ct method,  $NE = 1 / ((PE_{target}^{Ct,target}) / (PE_{reference}^{Ct,reference}))$  (PE = primer efficiency; Ct = cycle threshold; reference = *Actin*; target = *PI-Ilf*). The PEs were determined by fitting a linear regression line on the Ct-values of a qPCR on a standard cDNA dilution series. The qPCR primers for *PI-Ilf* (GB: AY129402.1, SGN/ITAG2.3: Solyc03g020080.2.1) [18,19] were: Fwd GACAAGTACTAGTAATCAATTATCC and Rev GGGCATATCCCGAACCAAGA. We used *Actin* (GB: XM.004235020.1, SGN/ITAG2.3: Solyc03g078400.2.1) as a reference for normalization using the following qPCR primers: Fwd TTAGCACCTTCCAGCAGATGT and Rev AACAGACAGGACACTCGCACT.

### 2.9. Spider mite egg-predation and predatory mite oviposition

We assessed (1) *P. longipes* predation of spider mite-eggs produced on *def-1* leaflets treated with tap water, tap water with 1 mM Ile or tap water with 0.05 mM JA + 1 mM Ile (as described above) and (2) the subsequent oviposition of the predator after eating these prey-eggs. To do so, we first allowed 15 adult female spider mites (*T. urticae* or *T. evansi*) to eat and produce eggs for 48 h on these *def-1* leaflets. Then, we removed the adult spider mites and counted their eggs. Subsequently, we transferred one *P. longipes* female from the cohort to these leaflets and assessed how many spider mite eggs they consumed within 48 h. At this stage we did not assess predatory mite oviposition since these mites had been feeding on cucumber spider mites shortly before (and possibly still digesting that material which could influence oviposition). To assure that the eggs laid by the predators were influenced by the treatments tested here we then, transferred these same predatory mites to freshly prepared *def-1* leaflets in water, Ile or JA + Ile with spider mite eggs. On this second batch of leaflets we assessed the oviposition of these predatory mites during 48 h, i. e. they had been feeding on spider mite eggs for 96 h in total, but only the number of eggs laid in the last 48 h were considered. Occasionally, predatory mites got stuck in the glue barrier or escaped from the leaflet and these were



**Fig. 4.** The relationship between JA-Ile and the reproductive performance of spider mites on tomato *def-1*. Leaflets of the JA-biosynthesis mutant *def-1* had been placed in tap water (“Water”), tap water + Ile (“Ile”) or tap water + JA + Ile (“JA + Ile”) for 24 h. Then 15 adult *T. evansi* and *T. urticae* females were introduced and were allowed to feed for 48 h. Subsequently, the reproductive performance of the mites was assessed and *def-1* leaflets submitted to the above treatments were assayed for endogenous JA-Ile levels. Bars in panel a) represent the mean number of eggs per female per day  $\pm$ SE. Gray bars indicate the oviposition of *T. evansi* infested leaflets and white bars indicate the oviposition on *T. urticae* infested leaflets. Different uppercase letters indicate significant differences for the *T. evansi* treatment and lowercase for *T. urticae* ( $P < 0.05$ ). The dose-response curves in panel b) show the relationship between the *def-1* leaflet JA-Ile content (ng gFW<sup>-1</sup>) and the reproductive performance (eggs per female per day) of *T. evansi* (filled circles, dotted line) and *T. urticae* (open circles, solid line).

excluded from the analysis. Therefore, at the end of the experiment, we had different numbers of replicates in between treatments. This resulted in the following sample sizes: (a) predation of *T. evansi* eggs from *def-1* in water ( $n = 27$ ); *def-1* in Ile ( $n = 26$ ) and *def-1* in JA + Ile ( $n = 22$ ) times; (b) predation of *T. urticae* eggs from *def-1* in water ( $n = 25$ ); *def-1* in Ile ( $n = 23$ ) and *def-1* in JA + Ile ( $n = 21$ ); (c) predatory mite oviposition on *T. evansi* eggs from *def-1* in water ( $n = 14$ ); *def-1* in Ile ( $n = 21$ ) and *def-1* in JA + Ile ( $n = 13$ ) and (d) predatory mite oviposition on *T. urticae* eggs from *def-1* in water ( $n = 19$ ); *def-1* in Ile ( $n = 11$ ) times and *def-1* in JA + Ile ( $n = 12$ ). From these data we calculated egg predation and predatory mite’s net reproduc-

tive efficiency (described in the next section) as a measure of its efficiency in converting prey eggs into offspring.

### 2.9.1. Statistical analysis

The egg-predation rate was calculated as the average number of eggs eaten per female predatory mite during 48 h. Because “plant” and “time” did not have significant effects on predation, differences in the predation rate of *P. longipes* on spider mite eggs derived from the different treatments were redone using GLM with Poisson error distribution corrected for overdispersion. These data are presented in Fig. 5a.

After assessing the predatory mite oviposition (data not presented in figure) we estimated the net reproductive efficiencies of *P. longipes* on different prey diets by dividing the number of eggs that predatory mite had deposited on the last 48 h by the number of spider mite eggs the predator had eaten during the first 48 h and multiplying this by 100%. The net reproductive efficiency was log-transformed and differences between the two treatments were analyzed using GLM with a normal error distribution for *T. urticae* and *T. evansi* separately. These data are presented in Fig. 5b.

### 2.10. Predatory mite prey-preference assay

We assessed if predatory mites can discriminate between prey-eggs derived from spider mites that had been feeding on plants with induced or suppressed JA-defenses. To do so, *P. longipes* was offered a choice between spider mite eggs from *T. urticae* (defense inducer) or *T. evansi* (defense suppressor) produced on wild type leaflets or *def-1* leaflets and we assessed prey-egg consumption during 24 h. This assay was performed on a “neutral” substrate, i.e. on bean leaf discs (2.5 cm in diameter): first, we divided the bean leaf disc in two halves by placing a strip of wet cotton wool in the middle of the disc as a barrier after which we introduced 15 adult spider mites that had been feeding from normal wild type or *def-1* tomato leaflets for the previous two days to either side of the barrier. These mites were allowed to deposit eggs for 24 h on the bean discs: these eggs were predominantly derived from the previous tomato diet, because dietary effects on mite oviposition are known to persist for at least 2–3 days [46]. After 24 h, mites and some of the eggs were removed leaving a total 60 eggs on each disc half. Then, the barrier was removed and a predatory mite was allowed to prey on these eggs for 24 h after which the number of eggs consumed on each side of the leaf disc was assessed. These experiments were repeated twice in time and, in total, the predation choice test on *T. evansi* eggs was repeated 23 times and on *T. urticae* eggs 12 times. These data are presented in Fig. 6a and b, respectively.

#### 2.10.1. Statistical analysis

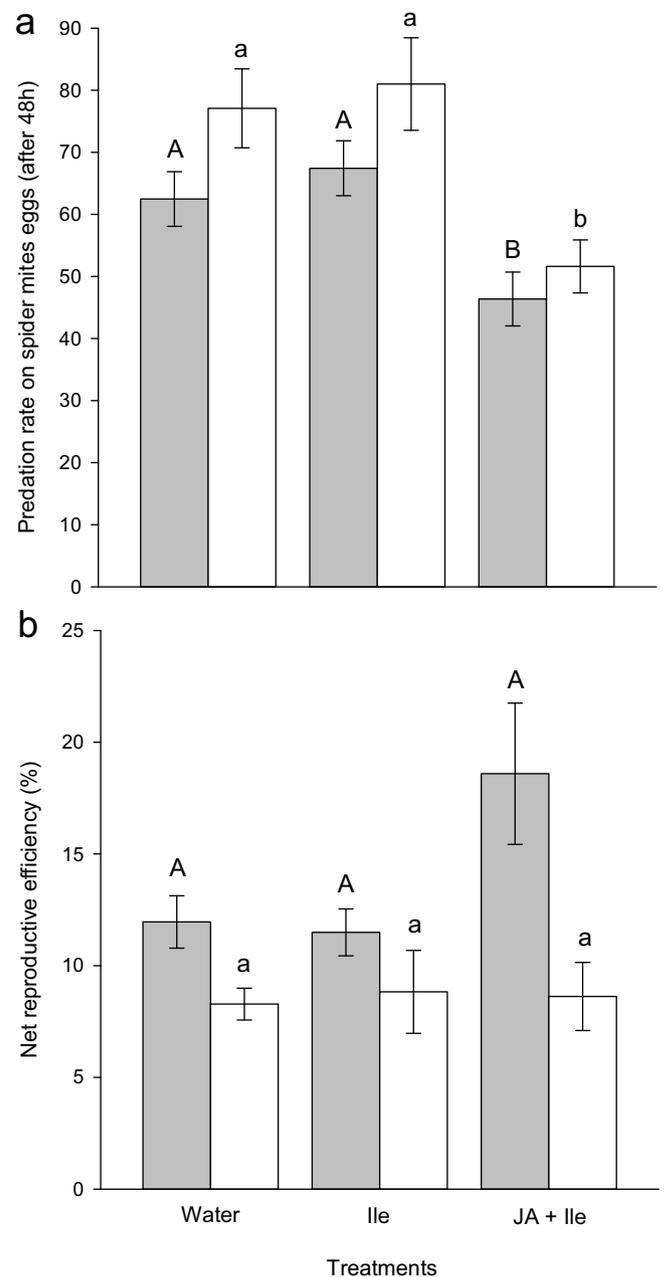
The differences in the predation rate of *P. longipes* on spider mite eggs derived from tomato wild type or *def-1* leaflets were analyzed separately for *T. evansi* and *T. urticae*. Due to the heterogeneity found among replicates in the predation choice test, these data did not fit into GLM with binomial error distribution and we used goodness-of-fit test [47] in Microsoft Excel.

## 3. Results

### 3.1. Spider mite performance on *def-1* tomato leaves treated with JA + Ile

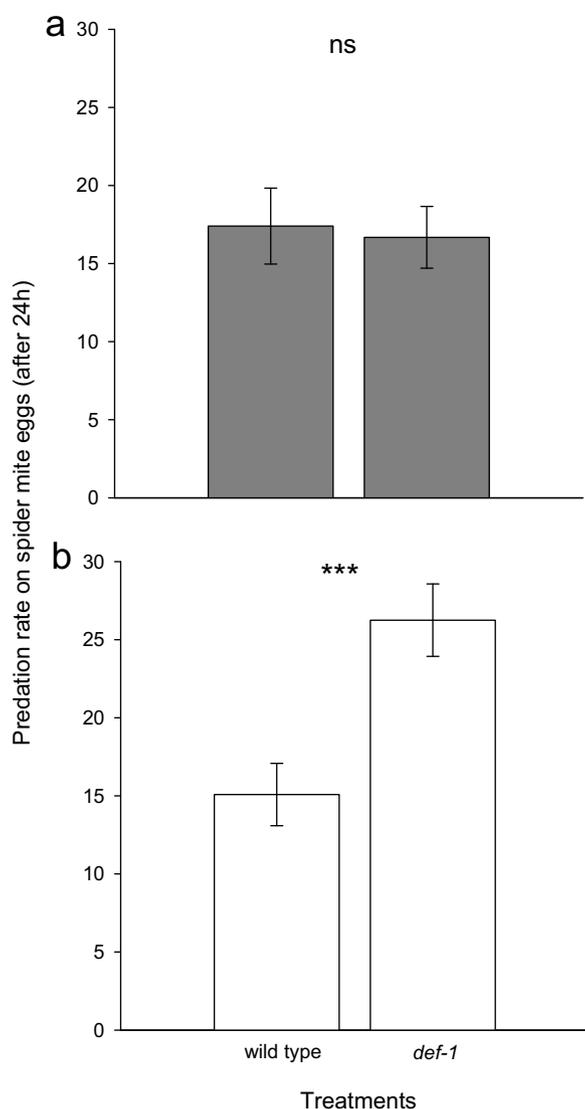
#### 3.1.1. JA + Ile treatment of *def-1*

The JA- response in detached *def-1* leaflets could be induced by using exogenous JA + Ile, which elevated their endogenous JA (Fig. 1a) and JA-Ile levels (Fig. 1b), as well as transcript levels of the JA-defense marker gene *PI-Ilf* (Fig. 2). Before conducting our experiments we had tested different concentrations of JA and Ile



**Fig. 5.** Predation rate and the net reproductive efficiency (%) of predatory mite *P. longipes* eating spider mite eggs derived from *def-1* leaflets treated with water or JA-Ile. Predatory mites were allowed to feed from *T. evansi* (gray bars) or *T. urticae* (white bars) eggs derived from *def-1* tomato leaflets treated either with tap water (“Water”), tap water + Ile (“Ile”) or with tap water containing 0.05 mM JA and 1 mM Ile (“JA + Ile”). Panel a) shows the number of spider mite eggs eaten per predatory mite during 48 h. Panel b) shows the predatory mite’s net reproductive efficiency, i.e. the number of spider mite eggs eaten per egg produced, calculated by dividing the number of offspring produced by the predators by the number of spider mite eggs eaten during 48 h  $\times$  100%. Different uppercase letters denote significant differences in *P. longipes* predation or net reproductive efficiency on *T. evansi* eggs ( $P < 0.05$ ) and lowercase letters represent significant differences in *P. longipes* predation or net reproductive efficiency on *T. urticae* eggs. Bars represent mean values  $\pm$  SE.

and found that adding 1 mM Ile to 0.05 mM JA roughly doubled the amount of JA-Ile in *def-1* within 24 h compared to when using only JA (data not shown). Prior to the experiments we had also investigated JA-Ile levels in the wild type tomato Castlemart plants infested with 15 spider mites for 2 days (Supplemental Fig. S1a) and with 250 spider mites for 6 days (Supplemental Fig. S1b). By comparing the JA-Ile levels in *def-1* leaflets treated with JA + Ile presented in Fig. 1b (around 25 ng gFW<sup>-1</sup>) with the naturally induced



**Fig. 6.** Predatory mite consumption of spider mite eggs derived from wild type and *def-1* tomato when these were offered simultaneously. Equal amounts of eggs from a) *T. evansi* derived from wild type or *def-1* tomatoes or from b) *T. urticae* derived from wild type or *def-1* were offered to single *P. longipes* females on a leaf disc of bean plant. The number of spider mite eggs consumed per female predatory mite was recorded after 24 h. Asterisks (\*\*\*) denote a significant difference ( $P < 0.001$ ) and (ns) indicates there was no significant difference ( $P > 0.05$ ). Bars represent the mean predation rate  $\pm$  SE.

levels presented in Supplemental Fig.S1b (around 5 ng gFW<sup>-1</sup> for *T. evansi* and 40 ng gFW<sup>-1</sup> for *T. urticae*) we argue that the treatment of JA + Ile had induced the JA-Ile levels in *def-1* leaflets similar to those reached by wild type plants infested with inducer spider mites. In addition, Fig. 1b shows that *def-1* leaflets treated with only water or with water + Ile control did not differ significantly for their endogenous JA-Ile levels (GLM:  $F_{[1,25]} = 1.93$ ;  $P = 0.18$  for *T. urticae*; GLM:  $F_{[1,21]} = 1.13$ ;  $P = 0.30$  for *T. evansi*), while JA-Ile levels in JA + Ile-treated *def-1* leaflets were significantly 10-fold higher than the levels in the water control (GLM:  $F_{[1,25]} = 58.5$ ;  $P < 0.001$  for *T. urticae*; GLM:  $F_{[1,21]} = 256.8$ ;  $P < 0.001$  for *T. evansi*). Similarly from what observed for JA-Ile levels, JA levels from water alone or the water + Ile control did not differ (GLM:  $F_{[1,26]} = 1.08$ ;  $P = 0.30$  for *T. urticae*; GLM:  $F_{[1,22]} = 1.15$ ;  $P = 0.29$  for *T. evansi*), but they differed from JA + Ile-treated *def-1* leaflets (GLM:  $F_{[1,27]} = 218.0$ ;  $P < 0.001$  for *T. urticae*; GLM:  $F_{[1,23]} = 512.9$ ;  $P < 0.001$  for *T. evansi*, Fig. 1a).

Applying JA+ Ile upregulated the expression of *PI-Ilf* roughly 200-fold (GLM:  $F_{[1,40]} = 612.4$ ;  $P < 0.001$ ) and it was independent from the mite species that infested the leaflet (GLM:  $F_{[1,39]} = 1.80$ ;  $P = 0.18$ ) (Fig. 2). The regression analysis of *PI-Ilf* and JA (Fig. 3a) as well as *PI-Ilf* and JA-Ile (Fig. 3b) confirmed that logarithmic NE of *PI-Ilf* in *def-1* leaflets infested with either mite species (JA: GLM:  $F_{[1,20]} = 160.6$ ;  $P < 0.001$  for *T. urticae*; GLM:  $F_{[1,19]} = 370.1$ ;  $P < 0.001$  for *T. evansi* and JA-Ile: GLM:  $F_{[1,20]} = 57.1$ ;  $P < 0.001$  for *T. urticae*; GLM:  $F_{[1,19]} = 26.4$ ;  $P < 0.001$  for *T. evansi*).

### 3.1.2. Spider mite performance on JA + Ile-treated *def-1*

Both *T. evansi* and *T. urticae* were found to lay significantly fewer eggs on *def-1* leaflets treated with JA-Ile than control leaflets (Fig. 4). *T. evansi* females produced roughly 1.5 eggs less per day (GLM:  $F_{[1,22]} = 5.4$ ;  $P = 0.034$ , Fig. 4a), while *T. urticae* females produced roughly 2 eggs less per day ( $F_{[1,26]} = 27.6$ ;  $P < 0.001$ , Fig. 4a) on leaflets treated with JA+ Ile compared to control leaflets. The oviposition of mites on *def-1* leaflets maintained in water or in water + Ile was similar for *T. evansi* (GLM:  $F_{[1,22]} = 0.86$ ;  $P = 0.36$ ) and slightly different for *T. urticae* (GLM:  $F_{[1,26]} = 4.3$ ;  $P = 0.05$ ).

Oviposition of spider mites was negatively correlated with JA-Ile levels (Fig. 4b). JA-Ile-oviposition dose-response curves shows that for both mite species reproductive performance drops by 30–40% and then, stabilizes despite increasing levels of JA-Ile (Fig. 4b). Performance of *T. evansi* reached its minimum at endogenous JA-Ile levels of around 5 ng gFW<sup>-1</sup>, while minimal performance of *T. urticae* was reached at levels around 10–15 ng gFW<sup>-1</sup>.

## 3.2. Spider mite egg-predation and predatory mite oviposition

### 3.2.1. Spider mite egg-predation

The predatory mite *P. longipes* consumed nearly a third less *T. urticae* eggs (GLM:  $\text{Chi}_{[1,67]}^2 = 9.4$ ;  $P = 0.003$ ) and a quarter less *T. evansi* eggs (GLM:  $\text{Chi}_{[1,73]}^2 = 7.0$ ;  $P = 0.009$ ) when these came from JA+ Ile treated *def-1* leaflets compared to eggs that came from control leaflets (Fig. 5a). Egg predation on eggs from the water control compared to eggs from the water + Ile control did not differ significantly (GLM:  $\text{Chi}_{[1,67]}^2 = 0.19$ ;  $P = 0.66$  for *T. urticae* and GLM:  $\text{Chi}_{[1,73]}^2 = 0.60$ ;  $P = 0.43$  for *T. evansi*).

### 3.2.2. Predatory mite's efficiency in converting prey eggs into offspring

Predators consumed less spider mite eggs when these came from JA + Ile treated *def-1* compared to the controls (Fig. 5a). Since the net reproductive efficiency of the predatory mites was not different after eating spider mite eggs from JA+ Ile-treated *def-1* leaflets compared to the controls (GLM:  $F_{[1,45]} = 2.34$ ;  $P = 0.10$  for *T. evansi* and GLM:  $F_{[1,39]} = 0.07$ ;  $P = 0.93$  for *T. urticae*) (Fig. 5b), the difference found on predatory mite oviposition under *T. urticae* eggs can be attributed solely to the lower egg consumption.

### 3.3. Predatory mite prey-preference assay

We offered *P. longipes* a choice between eggs from *T. evansi* that were either produced on wild type plants or on *def-1* tomato plants. The predators did not discriminate between these eggs since they consumed similar amounts ( $G_p = 0.3$ ;  $df = 1$ ;  $P = 0.59$ ) (Fig. 6a). When we offered *P. longipes* a choice between eggs from *T. urticae* that had been produced either on wild type or on *def-1* plants, the predators consumed more eggs derived from *def-1* ( $G_p = 36.7$ ;  $df = 1$ ;  $P < 0.001$ ) (Fig. 6b).

#### 4. Discussion

Here we showed that induction of defenses by JA in tomato has a negative impact on spider mite performance (Fig. 4a) with a maximum reduction of 30–40% at endogenous JA-Ile levels of around 10 ng gFW<sup>-1</sup>, but with no further reduction at higher JA-Ile levels (Fig. 4b). Furthermore, we showed that spider mite-egg consumption by *P. longipes* was lower when eggs came from *def-1* leaflets in which JA-defenses had been artificially induced as compared to non-treated control *def-1* leaflets (Fig. 5a). However, the net reproductive efficiency of the predatory mites was equal on all prey-egg diets. Finally, we showed that predatory mites discriminated between spider mite-eggs produced on tomato plants with and without induced JA-defenses (Fig. 6).

It has often been shown that exogenous application of JA can activate induced direct defenses in several plant species, including tomato [48–51]. The endogenous amounts of JA-Ile we retrieved from *def-1* leaflets when treated with 0.05 mM JA + 1 mM Ile ranged from 10 to 40 ng gFW<sup>-1</sup> and, hence, were comparable to those found in induced wild type tomato leaflets infested with the inducer spider mite *T. urticae* (Supplemental Fig. S1b) and similar to, albeit higher than, the levels we reported earlier for mite-induced tomato plants, i.e. 5 ± 3 ng gFW<sup>-1</sup> [21] and 13 ± 5 ng gFW<sup>-1</sup> [30]. The endogenous amount of JA we obtained in our JA+ Ile treated *def-1* leaflets exceeded those usually found in tomato plants infested by the same *T. urticae* strain that we used here 100–200 fold [21,30]. The levels of JA in tomato leaves reported across literature have wide range, i. e. from 0 to 2500 ng gFW<sup>-1</sup>, and differ greatly across cultivars [21,30,52,53]; the tissues that were sampled, e.g. roots [52], stem [54] or leaves [52]; if the plants were mechanically damaged or infested by (different types of) herbivores [55,56] or elicitor treatment [57]. Because we administered JA into the leaflets via the water in which they were placed, this compound could be taken up continuously through the petiolules, which is probably why we obtained such high endogenous levels. To verify that, we sampled vein and lamina of JA+ Ile treated *def-1* leaflets separately and we found around 60% of the JA back in the veins and 40% in lamina (Supplemental Fig. S2), i. e. most of the JA is indeed stored in the veins. In addition, the decrease in spider mite performance occurs when JA-Ile levels range from 2 to 15 ng gFW<sup>-1</sup> which is similar to the induced levels of JA-Ile in leaflets of wild type plants, i.e. between 2–40 ng gFW<sup>-1</sup> (Supplemental Fig. S1) and 5–13 ng gFW<sup>-1</sup> [21,30]. This and the fact that it was often shown before that JA-Ile, and not JA, is the active substance that binds to the receptor protein COI1 and JAZ protein triggering the downstream defense response [16,17] strongly suggests that the JA + Ile treatment gives rise to a defense response in *def-1* similar to that of wild type plants despite the relatively high JA levels.

Our JA+ Ile treatment of *def-1* shows that the defense-inducing *T. urticae*, as well as the defense-suppressing *T. evansi*, are both sensitive to JA-defenses and suggests that *T. evansi* is unable to suppress the JA-response in JA-Ile-treated *def-1*. This may be unexpected since *T. evansi* predominantly suppresses these defenses downstream of JA-Ile accumulation [21]. However, the same was observed for suppressor *T. urticae* mites on transgenic plants that display exceptionally strong JA-defenses due to an overexpressed prosystemin gene [15]. Possibly, the constant inflow of a relatively high amount of JA-Ile simply overpowers this mite's ability to down-regulate defense gene expression. Whatever the exact mechanistic reason, it provided us with the opportunity to assess the quantitative relationship between mite fitness and the magnitude of plant defenses.

Although negative effects of JA-defenses on herbivores such as *T. urticae* have been reported [15,21,30] the defense-response window for the oviposition of *T. evansi* appeared to be different from the oviposition of *T. urticae*, indicating that *T. evansi* is more

susceptible to JA defenses than *T. urticae*. Moreover, our data suggests that induction of plant defenses to concentrations higher than 10 ng gFW<sup>-1</sup> do not seem to further decrease the performance of both mite species, i.e. responses observed in wild type plants are optimal in the sense that a stronger response would not have increased resistance. However, there is considerable variation among individual mites in their response to JA-defenses. Hence, we feel one should be cautious drawing conclusions regarding plant resistance on the basis of the magnitude of a defense response, since clearly not all individuals of an herbivore population are similarly susceptible.

Not much is known about the exact downstream JA-inducible defenses that enable a plant to resist spider mites. For example, although JA-Ile accumulation correlated positively with *PI-II* marker-gene expression (Fig. 3) and negatively with spider mite reproductive performance (Fig. 4) the actual effects of PI-II proteins on spider mite survival and reproduction have not been evaluated explicitly. It was often found that the activity of chymotrypsin PI in tomatoes correlates negatively with mite performance, but it is unclear if this class of PI's is directly active against them, since their target proteases may not be present in the mite gut [58]. To our knowledge only two other classes of compounds have been associated with anti-spider mite defenses in tomato: methyl ketones [26] and the terpene 7-epi-zingiberene [25]. Cultivated tomatoes do not have either. Still, tomatoes produce many other putative defensive secondary metabolites, i.e. nitrogen-containing compounds like alkaloids/saponins [59–61], phenolics [62,63] and terpenoids [20,25], which are functionally and regulatory intertwined [64], as well as suite of defense-associated proteins [65–67] that could promote resistance to mites. Hence, which tomato defenses are responsible for the observed decrease in mite reproductive performance is yet to be determined.

There are some indications that spider mites can sequester plant toxins [68,69] and our data suggest that spider mites also transfer plant-related defensive products to their eggs such that these interfere with predation. Possibly, these substances pass through intact or are modified within the mite's gut system before they end up in the eggs. Sequestration of plant toxins has been reported before for insects and not only in their feeding stages [70,71] but also in their eggs [72,73], thereby acting as protection against natural enemies [74–77]. For example, Ferguson et al. [72] showed that labelled cucurbitacin B is stored not only in the hemolymph, but also in the body and eggs of five diabrotica species and Tallamy et al. [78] showed that females transferred 79% of the sequestered cucurbitacin during mating into their eggs. In addition, Eisner and Eisner [79] and Eisner et al. [80] found alkaloids in the eggs of the bella moth *Utethesia ornatrix*, while Tooker and De Moraes [81] showed that even JA can be stored in the eggs of several insect species belonging to eight distinct orders. Besides mediating changes in plant secondary metabolism, JA pathway mediates changes in plant primary metabolism [82] and therefore, we do not rule out the possibility that treating *def-1* tomato leaflets with JA+ Ile influenced besides defense, also the nutritional quality of those leaflets. There is a vast literature showing that the nutritional quality of plants decreases in response to herbivory [83] which in turn can affect not only the performance and quality of herbivores feeding on them, but also of natural enemies feeding on these herbivores. Whether our JA+ Ile treatment influenced the nutritional quality of the leaflets still needs to be investigated, but it could be that changes in plant quality alter also the quality of the spider mites feeding on them. Therefore, to trace which plant components spider mites transferred to their eggs after feeding on JA-Ile-treated *def-1* leaflets is an important step to understand their role in modulating predator preference and performance. With this end, we are currently working on techniques to allow us to sample eggs and

investigate their content, but so far we have not been successful yet.

Our experiments showed that the predatory mite *P. longipes* ate fewer eggs of spider mites that had been feeding on JA+ Ile treated *def-1* leaflets compared to control *def-1* leaflets (Fig. 5a). In addition, predators that had consumed *T. urticae* eggs from JA-Ile-treated *def-1* plants produced fewer eggs themselves, although their net reproductive efficiency was not affected (Fig. 5b). This suggests that these predators stopped eating, or ate at a lower rate, *T. urticae*-eggs from JA-Ile-treated *def-1* plants. Hence, JA-defenses may not only constrain predation, but also decrease a predator's net fecundity. Something similar, albeit not on eggs, was observed by Thaler et al. [84] who showed that the parasitoid *Hyposoter exiguae* developed slower and gained less weight on *Spodoptera exigua* caterpillars reared on JA-induced tomato plants than on caterpillars reared on non-induced tomato plants. However, treating wild type tomato plants with JA increased caterpillar parasitization in the field, possibly due to increased emission of volatiles, which in turn may have attracted more parasitoids to those plants. This shows that natural enemies can be affected by the JA induced defenses.

In the choice experiments between prey-eggs derived from wild type or from *def-1* plants, the predatory mites did not discriminate between eggs from *T. evansi* produced on wild type or *def-1* plants, whereas they preferred *T. urticae* eggs from *def-1* to those from wild type plants (Fig. 6). Probably, suppression of JA-defenses by *T. evansi* in wild type plants [21,24] may cause these eggs to be just as suitable for predators as eggs derived from *def-1*. In contrast, *T. urticae* induces JA-defenses only in wild type leaflets and this may be the reason for the predator's preference for eggs derived from *def-1* when offered a choice. In theory, in the field *T. urticae* could, therefore, benefit from residing on the same leaves or plants as *T. evansi* for two reasons: (i) its reproductive success could increase due to suppressed defenses [21] and (ii) it may escape predation more when natural enemies prefer to eat *T. evansi* eggs. However, the fact that natural *T. urticae* populations are currently being displaced by *T. evansi* on several host plants in Southern Europe [85], suggests that the potential benefits for *T. urticae* do not suffice to outcompete *T. evansi*. Furthermore, the predicted vulnerability of *T. evansi* to predation due to suppression of plant defenses clearly does not make it more easy to control by most of the commercially available predatory mites, which do not like to eat *T. evansi* eggs for unknown reasons [33,34].

Our study highlights the versatile role of JA defenses in shaping the interactions among plants, herbivorous mites, their offspring and predatory mites. The increase in plant toxicity for resistance-breeding purposes can lead to a decrease in egg predation. If this, consequently, interferes with the ability of predators to repress a prey population requires a study that spans multiple generations of both species. If so, this could have consequences for the compatibility of plant resistance-breeding or pest-control programs that make use of synthetic defense-elicitors [86] in conjunction with biological control agents. In this case, although *P. longipes* has been shown to be suitable for biological control of *T. evansi*, its ability to control this pest would work suboptimal in resistant-breeding crops. Finally, whether defense suppression by herbivores may evoke increased predation in nature remains to be determined. Possibly, suppressors of JA-defenses also suppress the attraction of natural enemies thereby compensating for the increased predation effect we observed. However, Sarmiento et al. [24] showed that *P. longipes* is attracted to tomato plants infested with *T. evansi*. Therefore, comparative field experiments using artificially induced tomatoes and wild type tomatoes infested by *T. evansi* in the presence of their natural enemies will be needed to determine how these interactions play out.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.plantsci.2016.08.004>.

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