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Role of Methyl Salicylate on Oviposition Deterrence in *Arabidopsis thaliana*

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Abstract Plants attacked by herbivores have evolved different strategies that fend off their enemies. Insect eggs deposited on leaves have been shown to inhibit further oviposition through visual or chemical cues. In some plant species, the volatile methyl salicylate (MeSA) repels gravid insects but whether it plays the same role in the model species *Arabidopsis thaliana* is currently unknown. Here we showed that *Pieris brassicae* butterflies laid fewer eggs on *Arabidopsis* plants that were next to a MeSA dispenser or on plants with constitutively high MeSA emission than on control plants. Surprisingly, the MeSA biosynthesis mutant *bsmt1-1* treated with egg extract was still repellent to butterflies when compared to untreated *bsmt1-1*. Moreover, the expression of BSMT1 was not enhanced by egg extract treatment but was induced by herbivory. Altogether, these results provide evidence that the deterring activity of eggs on gravid butterflies is independent of MeSA emission in *Arabidopsis,* and that MeSA might rather serve as a deterrent in plants challenged by feeding larvae.

Keywords Oviposition · *Pieris brassicae* · Methyl salicylate · *Arabidopsis thaliana*

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Introduction

Plant volatiles play a preponderant role in plant ecology where they serve, among other roles, to inform the surrounding organisms of the plant's physiological status. In response to herbivory, plants trigger complex direct and indirect defenses that ward off their enemies (Howe and Jander 2008; Mithöfer and Boland 2012; Wu and Baldwin 2010). As indirect defenses, attacked plants emit a blend of volatiles that attract parasitoid wasps and insect predators (Dicke and Baldwin 2010). Oviposition by phytophagous insects is known to be tightly dependent on the chemistry of the host plant (Hilker and Meiners 2011; Renwick and Chew 1994). For instance, plant volatiles are used by gravid insects to detect suitable substrates for oviposition (Rothschild and Schoonhoven 1977). In contrast, the presence of eggs deters butterflies from further oviposition. This behavior is linked to visual and chemical cues from either eggs or plants (Bergström et al. 1994; Blaakmeer et al. 1994a; de Vos et al. 2008; Renwick and Chew 1994; Rothschild and Schoonhoven 1977; Schoonhoven et al. 1981).

We recently discovered that *Arabidopsis thaliana* reacts to *Pieris brassicae* oviposition by accumulating salicylic acid (SA), a signal molecule that is essential for defense against fungal and bacterial pathogens (Bruessow et al. 2010). Early detection of egg-associated elicitors triggers a response similar to basal innate immunity, with the production of reactive oxygen species, callose deposition, local cell death, and

activation of the SA pathway, leading to the expression of defense genes (Gouhier-Darimont et al. 2013; Little et al. 2007; Reymond 2013). This finding was unexpected since feeding larvae are known to activate the jasmonic acid (JA) pathway, which is essential for an efficient defense against herbivory (Howe and Jander 2008; Reymond et al. 2004). Accordingly, the transcriptome of oviposited *Arabidopsis* plants was strikingly different from plants challenged with feeding larvae (Little et al. 2007).

Interestingly, methyl salicylate (MeSA) is a common volatile derived from SA through methylation by the enzyme BSMT1, and was shown to repel aphids, moths and thrips in soybean, *Brassica napus*, and cucumber, respectively (Koschier et al. 2007; Mallinger et al. 2011; Ulland et al. 2008). This volatile is released after herbivory in *Arabidopsis* and tomato (Ament et al. 2004; Chen et al. 2003; Snoeren et al. 2010a; Van Poecke et al. 2001) but its role in response to oviposition has never been assessed. In addition, BSMT1 has been shown to metabolize other substrates, including benzoic acid, *m*-hydroxybenzoic acid, and anthranilic acid (Chen et al. 2003).

Because *P. brassicae* oviposition on *Arabidopsis* induces the accumulation of SA, we reasoned that MeSA could be produced and deter future oviposition by butterflies. As eggs are inert and represent a non-immediate threat to the plant, this could represent an early response to avoid further increase in egg load.

Methods and Materials

Plant and Insects Growth Conditions All experiments were carried out in *Arabidopsis thaliana* Columbia ecotype (Col-0) background. Plants were grown in soil in growth chambers under short day conditions (8 hr light, 20 °C, 65 % relative humidity, 100 μmol m^{-2} s^{-1}). The soil consisted of 65 % humus, 10 % sand, 15 % perlite, and 10 % silt, and was not complemented with fertilizer. The *bsmt1-1* mutant (SALK_140496) was provided by Jürgen Zeier (University of Duesseldorf), and OsS6 was obtained from Yang Do Choi (Seoul National University). Individuals of *P. brassicae* were reared on *Brassica oleracea* var *gemmifera* in a greenhouse (Reymond et al. 2000).

Oviposition Dual Choice Assays with P. brassicae Butterflies Four- to 5-wk-old plants were used for choice assays. All experiments were performed in a greenhouse under constant light. Three female and two male butterflies were placed in insect tents (60×60×60 cm, Bugdorm, Taiwan) with four plants of each treatment. During the experiment, butterflies were allowed to mate and drink sugary water. The number of eggs laid was assessed after 12 hr. Methyl salicylate (Sigma-Aldrich, purity >99 %) was diluted in hexane to a final concentration of 0.15 mg/μl, and 5 μl were applied to a volatile dispenser consisting of half cotton swabs disposed at the center of the pot. Hexane was applied to the cotton swabs of control plants. The solvent was allowed to evaporate before beginning the experiment. The amount of MeSA used in the experiment was shown to repel *Mamestra brassicae* moths and corresponds to a release rate of 50–100 ng/hr (Ulland et al. 2008).

Pieris brassicae eggs laid on cabbage leaves were crushed with a pestle in Eppendorf tubes. After centrifugation (15′ 000 g, 3 min), the supernatant ("egg extract") was stored at −20 °C. For egg-extract treatment, 2×2 μl of egg extract were applied to the abaxial surface of two leaves per plant for 3 days.

Each comparison was performed several times in parallel, and replicated on different days. Data were analyzed comparing the number of eggs laid on each genotype/treatment using a *Generalized Linear Model* (*GLM*) controlling for tent and temporal effects.

Quantitative Real-Time PCR (QPCR) Egg-extract treatment was performed by applying 2×2 μl of *P. brassicae* egg extract on two leaves per plant for 5 days. For herbivory treatment, two *P. brassicae* neonates were placed on each of four *Arabidopsis* plants and allowed to feed for 2 days. Tissue samples from local leaves treated with egg-extract were ground in liquid nitrogen. Total RNA was extracted using a RNeasy Plant Mini kit and treated with DNaseI according to the manufacturer's instructions (Qiagen). cDNA was synthesized from 500 ng of total RNA using M-MLV reverse transcriptase (Invitrogen), and diluted eightfold with water. Quantitative real-time PCR reactions were performed using Brilliant III Fast SYBR-Green QPCR Master Mix on a Mx3000P real-time PCR instrument (Agilent) with the following program: 95 °C for 3 min, then 40 cycles of 10 sec at 95 °C, 20 sec at 60 °C. Values were normalized to the housekeeping gene *EIF4A1* (At3g13920). The expression level of a target gene (TG) was normalized to the reference gene (RG) and calculated as normalized relative quantity (NRQ) as follows: $NRQ = E^{CtRG}/E^{CtTG}$. Primer efficiencies (E) were evaluated by a five-step dilution regression. For each experiment, three biological replicates were analyzed. Different genes analyzed were amplified using the following primers: *BSMT1* (AT3G11480) fwd 5′-CATTCAACATGCCGTTTTATG–3′ and rev 5′-CATTGGTTCACTAACAGCTC-3′; *PR-1* (AT2G14610) fwd 5′-GTGGGTTAGCGAGAAGGCTA-3′ and rev 5′- ACTTTGGCACATCCGAGTCT-3′; *EIF4A1* (At3g13920) fwd 5′-CCAGAAGGCACACAGTTTGA-3′ and rev 5′-GACTGAGCCTGTTGAATCAC-3′.

Dynamic Headspace Collection and MeSA Analysis To verify MeSA emission by *Arabidopsis* with and without *P. brassicae* oviposition, headspace volatiles of individual plants enclosed

in glass bottles were collected in a volatile collection system (ARS, Gainesville, FL, USA). Airflow was regulated to 1.0 l/min, and volatiles were trapped using SuperQ adsorbent polymer (Alltech Associates Inc., Deerfield, IL, USA). Volatiles were collected for eight plants during 24 hr from 48 to 72 hr after *P. brassicae* oviposition. Twelve plants without oviposition were used as controls. Headspace collection and analysis were done as described previously (Peñaflor et al. 2011), with the following modifications: after injection, the column temperature was maintained at 40 °C for 3 min and then increased to 100 °C at 8 °C/min and subsequently to 220 °C at 5 °C/min followed by a postrun of 3 min at 250 °C. MeSA was identified by comparing its mass spectra and retention time with MeSA pure standard (Sigma-Aldrich, St. Louis, MO, USA) and with that of the NIST05 library.

Statistical Analyses All statistical analyses were carried out with R software version 3.0.1 (http://www.R-project.org).

Results

Egg Extract Treatment and MeSA Repel P. brassicae Butterflies The number of eggs laid on plants pretreated with *P. brassicae* egg extract, which mimics natural oviposition (Little et al. 2007), was significantly lower than on intact plants (Fig. 1a). When applied on a volatile dispenser placed next to *Arabidopsis* plants, MeSA decreased the total number of eggs laid compared to dispensers treated with solvent alone (Fig. 1b). Similarly, butterflies were repelled by *Arabidopsis* OsS6 mutant plants overexpressing a *BSMT1* homolog from rice (*Oryza sativa*) (Fig. 1c). OsS6 plants have been shown to emit MeSA constitutively, even in the absence of a stimulus (Koo et al. 2007). Given the repelling role of MeSA and previous observations that oviposited plants accumulate SA (Bruessow et al. 2010), which could be transformed to MeSA by BSMT1, we then tested whether MeSA accumulates after oviposition in *Arabidopsis*. However, we could not detect MeSA in plant volatiles collected between 48 and 72 hr after oviposition, with a detection limit <1 ng (Supplemental resource 1).

To further evaluate the involvement of MeSA we used the *Arabidopsis bsmt1-1* mutant that has no detectable MeSA emission (Attaran et al. 2009). Surprisingly, *bsmt1-1* plants treated with egg extract still repelled *P. brassicae* butterflies, as there were significantly more eggs laid on untreated than on treated *bsmt1-1* plants (Fig. 1d). Thus, our results indicate that MeSA emission is able to inhibit oviposition, but that this volatile is not responsible for egg extract-induced deterrence of ovipositing butterflies in *Arabidopsis*. However, when butterflies were given the choice between egg extract-treated Col-0 and egg extract-treated *bsmt1-1*, they significantly laid more eggs on Col-0 (Fig. 1e). Finally, butterflies also preferred non-treated Col-0 over non-treated *bsmt1-1*, suggesting that BSMT1 plays a role in attracting *P. brassicae* (Fig. 1f).

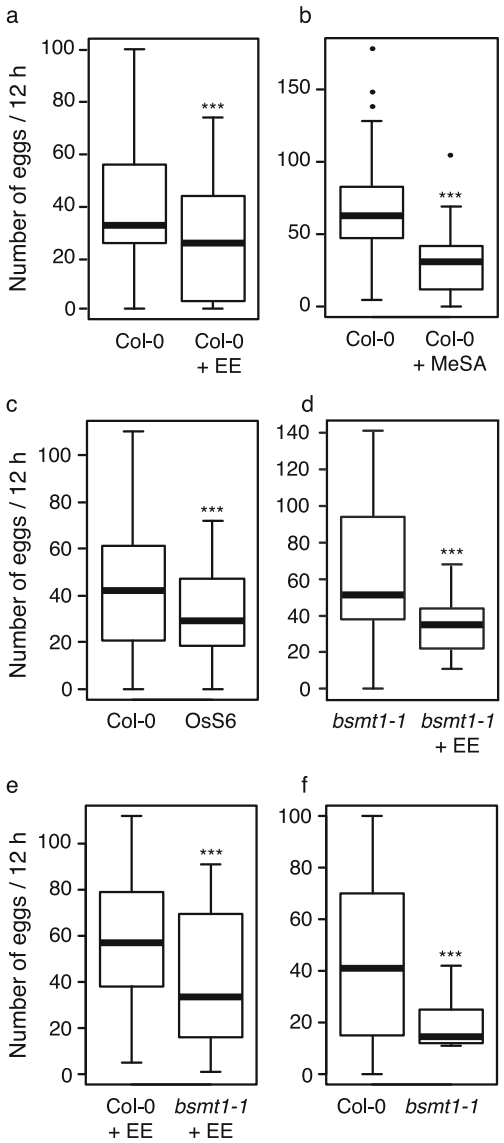

Fig. 1 Dual-choice oviposition tests with *Pieris brassicae*. Three female and two male *P. brassicae* butterflies were placed in a tent containing two groups of four *Arabidopsis* plants. The number of eggs laid was assessed after 12 hr of continuous light. Boxplots represent values from six to twenty-five biological replicates. **a** Number of eggs laid on untreated plants or plants pretreated with *P. brassicae* egg extract (EE) for 3 days. **b** Number of eggs laid on control plants or plants placed next to a MeSA dispenser. Methyl salicylate (0.15 mg/µl in hexane) was applied to a volatile dispenser disposed at the center of the pot. Control plants were placed next to a hexane dispenser. **c** Number of eggs laid on wild-type or *Arabidopsis* OsS6 line that overexpresses the rice BSMT1 gene. **d** Number of eggs laid on untreated or EE-treated *bsmt1-1* plants. **e** Number of eggs laid on EE-treated Col-0 or *bsmt1-1* plants. **f** Number of eggs laid on untreated Col-0 or *bsmt1-1* plants. Oviposition data were analyzed with a *Generalized Linear Model*. Asterisks indicate a significant difference compared to the control (*** $P<0.001$)

Expression of BSMT1 in Response to Herbivory To further explore whether MeSA emission was linked to oviposition, we analyzed the expression of *BSMT1* and *PR-1* in plants treated with egg extract or challenged with *P. brassicae* larvae. *PR-1* is a marker gene for the SA pathway, and it is induced by *P. brassicae* egg extract treatment (Gouhier-Darimont et al. 2013; Little et al. 2007). *PR-1* expression increased after egg extract treatment but not after herbivory, which is consistent with previous studies (Bruessow et al. 2010; Reymond et al. 2004) (Fig. 2a). In contrast, *BSMT1* expression was not induced after 5 days of egg extract treatment, but was strongly induced after herbivory by *P. brassicae* (Fig. 2). In support of this finding, BMST1 has been strongly upregulated by *P. rapae* feeding in *Arabidopsis* (Snoeren et al. 2010a). Moreover, egg extract treatment did not induce *BSMT1* after 24 , 48, or 72 hr (data not shown).

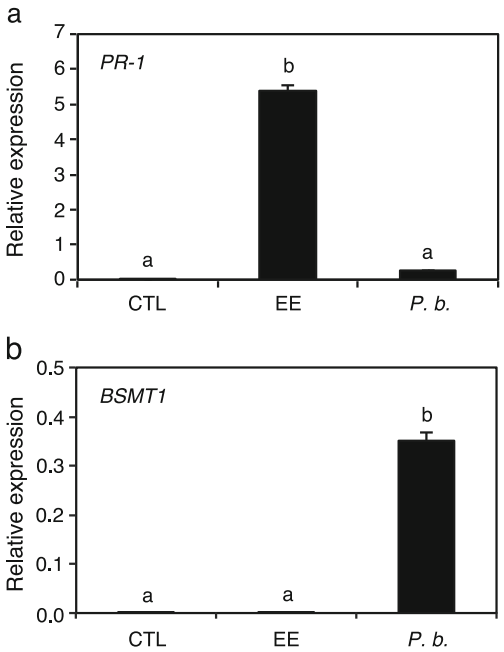

Fig. 2 Expression of *PR-1* **a** and *BSMT1* **b** after treatment with *Pieris brassicae* egg extract (EE) for 5 days or feeding by *P. brassicae* (*P. b.*) for 2 days. Expression levels were measured by QPCR and are relative to the housekeeping gene *EIF4A1*. Values are mean relative expression ± SE of three technical replicates. Similar results were obtained in two independent experiments. Different letters indicate significant differences at $P<0.05$ (Tukey's honest significant difference test)

Discussion

Pieris brassicae oviposition on plants pretreated with egg extract was lower than on untreated plants, confirming earlier observations with other plant species that butterflies can detect oviposited plants and avoid overloading (Blaakmeer et al. 1994a ; Shapiro 1981; Rothschild and Schoonhoven 1977). Although egg extract was applied on leaves facing the soil to avoid visual recognition by butterflies, this set-up did not prevent detection of egg-derived cues, suggesting that a chemical response was involved. Interestingly, our results using artificial dispensers and overexpressing lines show clearly that MeSA emission deters oviposition. Previous studies reported a similar effect for the moth *M. brassicae*, the thrips *Frankliniella occidentalis*, and the hemipteran pest *Lygus Hesperus* (Koschier et al. 2007; Ulland et al. 2008; Williams et al. 2010). However, *bsmt1-1* plants lacking MeSA were still able to repel butterflies when treated with egg extract, strongly suggesting that the deterring activity of eggs on gravid butterflies is independent of MeSA emission. In support of this finding, expression of *BSMT1*, which was reported to reflect MeSA emission in *Arabidopsis* (Snoeren et al. 2010a), was not induced by egg extract treatment. Moreover, we could not detect MeSA from the volatile blend of oviposited *Arabidopsis* plants. By comparison, OsS6 plants emit 6± 2 ng/g FW/ 24 hr (Koo et al. 2007), and *Arabidopsis* infected with *Pseudomonas syringae* pv. *maculicola* emit between 15 and 45 ng/g FW/ hr (Attaran et al. 2009), values that are well above the ca. 1 ng detection limit of our instrument. Furthermore, oviposition of *P. brassicae* has been shown to reduce MeSA emission in *Brassica oleracea* (Bergström et al. 1994). Finally, it also has been reported that plants treated with SA do not release MeSA (Koo et al. 2007). Collectively, these data provide strong evidence that MeSA is not involved in repelling butterflies after oviposition or treatment with egg extract.

Interestingly, we found that *bsmt1-1* plants received fewer eggs than wild-type plants in dual-choice experiments, irrespective of egg extract pre-treatment. This suggests that BSMT1 might have a positive role by producing a compound that attracts female butterflies. BSMT1 belongs to the SABATH family of methyl transferases, and *in vitro* analyses have shown that, besides SA, this enzyme catalyzes the methylation of benzoic acid, anthranilic acid, and *m*-hydroxybenzoic acid, with the highest activity towards benzoic acid (Chen et al. 2003). In addition, we noticed that *bsmt1-1* plants have longer petioles than Col-0 and display leaf epinasty (Supplementary resource 2). Thus, whether any of the methylated metabolites and/or *bsmt1-1* leaf phenotype influence butterflies for their choice of an oviposition site will need further investigation.

Evidence for the absence of a role for MeSA in response to oviposition as well as the observation that egg extract-treatment repelled butterflies implies that other factor(s) may render plants less acceptable for females. First, although egg extracts were applied underneath *Arabidopsis* leaves, we cannot formally exclude that visual factors informed butterflies about prior occupancy. Indeed, eggs or egg extract treatment cause chlorosis at the site of deposition in *Arabidopsis* Col-0 (Bruessow et al. 2010; Reymond 2013). An elegant experiment recently demonstrated that *Pieris rapae* butterflies could discriminate green *Arabidopsis* leaves from variegated greenwhitish leaves, obtained after silencing a phytoene desaturase

gene (Zheng et al. 2010). Alternatively, infochemicals from either the egg extract or the plant could be detected by gravid females. Avenanthramide alkaloids have been identified in eggs of *P. brassicae* and *P. rapae* and shown to inhibit oviposition when sprayed on cabbage leaves (Blaakmeer et al. 1994b). However, since the cabbage leaves were still repulsive after removal of *P. brassicae* eggs and the avenanthramides were no longer detectable, other plant chemicals were postulated to deter oviposition, although their nature has not been determined (Blaakmeer et al. 1994a). Glucosinolates (GS) are well-described defense compounds of the Brassicaceae (Halkier and Gershenzon 2006). Many crucifer specialists use GS as signals for oviposition, as larvae are able to detoxify them and thus feed unharmed on the plants (Hopkins et al. 2009; Huang and Renwick 1994; Renwick and Chew 1994). Induction of GS biosynthesis genes and GS accumulation are triggered by herbivory and are regulated by the JA pathway in *Arabidopsis* (Schweizer et al. 2013). Since *P. brassicae* eggs were shown to suppress the expression of JA-dependent defense genes in *Arabidopsis*, including GS-related genes (Bruessow et al. 2010), an intriguing hypothesis is that GS content might be reduced after oviposition, and therefore this would lower the attractiveness of *Arabidopsis* plants for further egg laying. In order to test these hypotheses, future studies should aim at measuring leaf chemical changes or emission of volatiles that follow oviposition in *Arabidopsis* and use biosynthesis mutants to identify deterring molecules.

Previous microarray experiments on plants oviposited or damaged by herbivory have reported that *BSMT1* was induced by *P. rapae* and *Spodoptera littoralis* feeding but not by eggs (Little et al. 2007; Reymond et al. 2004). We showed here by QPCR that *BMST1* is indeed not upregulated by egg extract treatment, whereas it is strongly induced by herbivory. MeSA emission has been reported to occur after herbivory in several plant species including *Arabidopsis*, (Snoeren et al. 2010a,b; Van Poecke et al. 2001), wild tobacco (Kessler and Baldwin 2001), maize (Turlings et al. 1998), rice (Zhao et al. 2010), cotton (Rodriguez-Saona et al. 2001), cucumber (Agrawal et al. 2002), and potato (Bolter et al. 1997). In support of these findings, BSMT1 transcript levels have been induced after methyl jasmonate (MeJA) application and herbivory in *Arabidopsis* (Chen et al. 2003; Snoeren et al. 2010a), indicating that MeSA emission is under the control of the JA-pathway.

Interestingly, the production/emission of MeSA as well as the expression of *BSMT1* also has been found to be induced after infection with the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis*. This effect is due to the presence of coronatine (COR), a bacterial effector that mimics JA-Ile, which is the bioactive JA (Attaran et al. 2009; Zheng et al. 2012). It would be interesting to carry out oviposition tests with plants inoculated with *P. syringae* to determine whether butterflies avoid infected plants, thus maximizing the survival of their progeny. Use of COR^- strains could confirm the role of this effector in oviposition responses.

In summary, our results suggest that MeSA emission is not responsible for reduced oviposition by *P. brassicae* on egg-treated *Arabidopsis* plants, but that it may rather play a role during larval feeding that blocks further oviposition. Whether this is a strategy developed by the plant that prevents an excess of attackers or by the insect that controls food availability for developing larvae deserves future study. A recent meta-analysis offered clear support for the preference-performance hypothesis, which states that female insects have evolved to oviposit more eggs on plants on which their offspring perform best (Gripenberg et al. 2010). This indicates that the avoidance of MeSA-emitting plants by females could be linked to a poorer performance on such plants.

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