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ROLE OF METHYL SALICYLATE ON OVIPOSITION DETERRENCE IN *Arabidopsis thaliana*

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

14

15 **Key Words** - Oviposition, *Pieris brassicae*, Methyl Salicylate, *Arabidopsis thaliana*.

16

17

## 1 INTRODUCTION

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

15 We recently discovered that *Arabidopsis thaliana* reacts to *Pieris brassicae*  
16 oviposition by accumulating salicylic acid (SA), a signal molecule that is essential for defense  
17 against fungal and bacterial pathogens (Bruessow et al. 2010). Early detection of egg-  
18 associated elicitors triggers a response similar to basal innate immunity, with the production  
19 of reactive oxygen species, callose deposition, local cell death, and activation of the SA  
20 pathway, leading to the expression of defense genes (Little et al. 2007; Gouhier-Darimont et  
21 al. 2013; Reymond 2013). This finding was unexpected since feeding larvae are known to  
22 activate the jasmonic acid (JA) pathway, which is essential for an efficient defense against  
23 herbivory (Reymond et al. 2004; Howe and Jander 2008). Accordingly, the transcriptome of  
24 oviposited *Arabidopsis* plants was strikingly different from plants challenged with feeding  
25 larvae (Little et al. 2007).

1            Interestingly, MeSA is a common volatile derived from SA through methylation by  
2 the enzyme BSMT1 and was shown to repel aphids, moths and thrips in soybean, *Brassica*  
3 *napus*, and cucumber, respectively (Koschier et al. 2007; Ulland et al. 2008; Mallinger et al.  
4 2011). This volatile is released after herbivory in *Arabidopsis* and tomato (Van Poecke et al.  
5 2001; Chen et al. 2003; Ament et al. 2005; Snoeren et al. 2010a) but its role in response to  
6 oviposition has never been assessed. In addition, BSMT1 was shown to metabolize other  
7 substrates, including benzoic acid, *m*-hydroxybenzoic acid, and anthranilic acid (Chen et al.  
8 2003).

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

13

#### 14 METHODS AND MATERIALS

15 *Plant and Insects Growth Conditions.* All experiments were carried out in *Arabidopsis*  
16 *thaliana* Columbia ecotype (Col-0) background. Plants were grown in soil in growth  
17 chambers in short day conditions (8 h light, 20° C, 65 % relative humidity, 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).  
18 The soil consisted of 65% humus, 10% sand, 15% perlite and 10% silt and was not  
19 complemented with fertilizer. The *bsmt1-1* mutant (SALK\_140496) was kindly provided by  
20 Jürgen Zeier (University of Duesseldorf), and OsS6 was obtained from Yang Do Choi (Seoul  
21 National University). Individuals of *Pieris brassicae* were reared on *Brassica oleracea* var  
22 *gemmifera* in a greenhouse (Reymond et al. 2000).

23

24 *Oviposition Dual Choice Assays with P. brassicae Butterflies.* Four- to five-week-old plants  
25 were used for choice assays. All experiments were performed in a greenhouse under constant

1 light. Three females and two males butterflies were placed in insect tents (60x60x60 cm,  
2 Bugdorm, Taiwan) with four plants of each treatment. During the experiment, butterflies were  
3 allowed to mate and drink sugary water. The number of eggs laid was assessed after 12 h.  
4 Methyl salicylate (Sigma-Aldrich, purity >99 %) was diluted in hexane to a final  
5 concentration of 0.15 mg/μl, and 5 μl was applied to a volatile dispenser consisting of half  
6 cotton swabs disposed at the center of the pot. Hexane was applied to the cotton swabs of  
7 control plants. The solvent was allowed to evaporate before beginning of the experiment. The  
8 amount of MeSA used in the experiment was shown to repel *Mamestra brassicae* moths and  
9 corresponds to a release rate of 50-100 ng/h (Ulland et al. 2008).

10 *P. brassicae* eggs laid on cabbage leaves were crushed with a pestle in Eppendorf  
11 tubes. After centrifugation (15'000 g, 3 min), the supernatant ("egg extract") was stored at -  
12 20°C. For egg-extract treatment, 2 x 2 μl of egg extract were applied to the abaxial surface of  
13 two leaves per plant for three days.

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

17

18 *Quantitative Real-Time PCR (QPCR)*. Egg-extract treatment was performed by applying 2 x 2  
19 μl of *P. brassicae* egg extract on two leaves per plant for five days. For herbivory treatment,  
20 two *P. brassicae* neonates were placed on each of four *Arabidopsis* plants and allowed to feed  
21 for two days. Tissue samples from local leaves treated with egg-extract were ground in liquid  
22 nitrogen. Total RNA was extracted using RNeasy Plant Mini kit and treated with DNaseI  
23 according to the manufacturer's instructions (Qiagen). cDNA was synthesized from 500 ng of  
24 total RNA using M-MLV reverse transcriptase (Invitrogen) and diluted eightfold with water.  
25 Quantitative real-time PCR reactions were performed using Brilliant III Fast SYBR-Green

1 QPCR Master Mix on a Mx3000P real-time PCR instrument (Agilent) with the following  
2 program: 95 °C for 3 min, then 40 cycles of 10 s at 95 °C, 20 s at 60 °C. Values were  
3 normalized to the housekeeping gene *EIF4A1* (At3g13920). The expression level of a target  
4 gene (TG) was normalized to the reference gene (RG) and calculated as normalized relative  
5 quantity (NRQ) as follows:  $NRQ = E^{C_{iRG}}/E^{C_{iTG}}$ . Primer efficiencies (E) were evaluated by  
6 five-step dilution regression. For each experiment, three biological replicates were analyzed.  
7 Different genes analyzed were amplified using the following primers: *BSMT1* (AT3G11480)  
8 fwd 5'-CATTCAACATGCCGTTTTATG-3' and rev 5'-CATTGGTTCACATAACAGCTC-  
9 3'; *PR-1* (AT2G14610) fwd 5'-GTGGGTTAGCGAGAAGGCTA-3' and rev 5'-  
10 ACTTTGGCACATCCGAGTCT-3'; *EIF4A1* (At3g13920) fwd 5'-  
11 CCAGAAGGCACACAGTTTGA-3' and rev 5'-GACTGAGCCTGTTGAATCAC-3'.

12  
13 *Dynamic Headspace Collection and MeSA Analysis.* To verify MeSA emission by  
14 *Arabidopsis* with and without *P. brassicae* oviposition, headspace volatiles of individual  
15 plants enclosed in glass bottles were collected in a volatile collection system (ARS,  
16 Gainesville, FL, USA). Airflow was regulated to 1.0 L/min and volatiles were trapped using  
17 SuperQ adsorbent polymer (Alltech Associates Inc., Deerfield, IL, USA). Volatiles were  
18 collected for eight plants during 24 h from 48 to 72 h after *P. brassicae* oviposition. Twelve  
19 plants without oviposition were used as controls. Headspace collection and analysis were  
20 done as described previously (Peñaflor et al. 2011), with the following modifications: after  
21 injection, the column temperature was maintained at 40 °C for 3 min and then increased to  
22 100 °C at 8 °C/min and subsequently to 220 °C at 5 °C/min followed by a postrun of 3 min at  
23 250 °C. MeSA was identified by comparing its mass spectra and retention time with MeSA  
24 pure standard (Sigma-Aldrich, St. Louis, MO, USA) and with that of the NIST05 library.

25

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

3

#### 4 RESULTS

5 *Egg Extract Treatment and MeSA Repel P. brassicae Butterflies.* The number of eggs laid on  
6 plants pretreated with *P. brassicae* egg extract, which mimics natural oviposition (Little et al.  
7 2007), was significantly lower than on intact plants (Fig. 1a). When applied on a volatile  
8 dispenser placed next to *Arabidopsis* plants, MeSA decreased the total number of eggs laid  
9 compared to dispensers treated with solvent alone (Fig. 1b). Similarly, butterflies were  
10 repelled by *Arabidopsis* OsS6 mutant plants overexpressing a *BSMT1* homolog from rice  
11 (*Oryza sativa*) (Fig. 1c). OsS6 plants have been shown to emit MeSA constitutively, even in  
12 the absence of stimulus (Koo et al. 2007). Given the repelling role of MeSA and previous  
13 observations that oviposited plants accumulate SA (Bruessow et al. 2010), which could be  
14 transformed to MeSA by BSMT1, we then tested whether MeSA accumulates after  
15 oviposition in *Arabidopsis*. However, we could not detect MeSA in plant volatiles collected  
16 between 48 and 72 h after oviposition, with a detection limit < 1 ng (Online resource 1).

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

1 butterflies also preferred non-treated Col-0 over non-treated *bsmt1-1*, suggesting that BSMT1  
2 plays a role in attracting *P. brassicae* (Fig. 1f).

3  
4 *Expression of BSMT1 in Response to Herbivory.* To further explore whether MeSA emission  
5 was linked to oviposition, we analyzed the expression of *BSMT1* and *PR-1* in plants treated  
6 with egg extract or challenged with *Pieris brassicae* larvae. *PR-1* is a marker gene for the SA  
7 pathway and is induced by *P. brassicae* egg extract treatment (Little et al. 2007; Gouhier-  
8 Darimont et al. 2013). *PR-1* expression increased after egg extract treatment but not after  
9 herbivory, which is consistent with previous studies (Reymond et al. 2004; Bruessow et al.  
10 2010) (Fig. 2a). On the contrary, *BSMT1* expression was not induced after five days of egg  
11 extract treatment but was strongly induced after herbivory by *P. brassicae* (Fig. 2). In support  
12 of this finding, *BMST1* was strongly upregulated by *Pieris rapae* feeding in *Arabidopsis*  
13 (Snoeren et al. 2010a). Moreover, egg extract treatment did not induce *BSMT1* after 24 h, 48  
14 h, and 72 h (data not shown).

15

## 16 DISCUSSION

17 *P. brassicae* oviposition on plants pretreated with egg extract was lower than on untreated  
18 plants, confirming earlier observations with other plant species that butterflies can detect  
19 oviposited plants and avoid overloading (Rothschild and Schoonhoven 1977; Shapiro 1981;  
20 Blaakmeer et al. 1994a). Although egg extract was applied on leaves facing the soil to avoid  
21 visual recognition by butterflies, this set-up did not prevent detection of egg-derived cues,  
22 suggesting that a chemical response was involved. Interestingly, our results using artificial  
23 dispensers and overexpressing lines clearly show that MeSA emission deters oviposition.  
24 Previous studies reported a similar effect for the moth *M. brassicae*, the thrips *Frankliniella*  
25 *occidentalis*, and the hemipteran pest *Lygus Hesperus* (Koschier et al. 2007; Ulland et al.



1 2008; Williams et al. 2010). However, *bsmt1-1* plants lacking MeSA were still able to repel  
2 butterflies when treated with egg extract, strongly suggesting that the deterring activity of  
3 eggs on gravid butterflies is independent of MeSA emission. In support of this finding,  
4 expression of *BSMT1*, which was reported to reflect MeSA emission in *Arabidopsis* (Snoeren  
5 et al. 2010a), was not induced by egg extract treatment. Moreover, we could not detect MeSA  
6 from the volatile blend of oviposited *Arabidopsis* plants. By comparison, OsS6 plants emit  
7  $\pm 2$  ng/g FW/ 24 h (Koo et al. 2007) and *Arabidopsis* infected with *Pseudomonas syringae* pv.  
8 *maculicola* emit between 15 to 45 ng/g FW/ h (Attaran et al. 2009), values that are well above  
9 the ca. 1 ng detection limit of our instrument. Furthermore, oviposition of *P. brassicae* even  
10 reduced MeSA emission in *Brassica oleracea* (Bergström et al. 1994). Finally, it was also  
11 reported that plants treated with SA do not release MeSA (Koo et al. 2007). Collectively,  
12 these data provide strong evidence that MeSA is not involved in repelling butterflies after  
13 oviposition or treatment with egg extract.

14 Interestingly, we found that *bsmt1-1* plants received fewer eggs than wild-type plants  
15 in dual-choice experiments, irrespective of egg extract pre-treatment. This suggests that  
16 BSMT1 might have a positive role by producing a compound that attracts female butterflies.  
17 BSMT1 belongs to the SABATH family of methyl transferases and *in vitro* analyses have  
18 shown that, besides SA, this enzyme catalyzes the methylation of benzoic acid, anthranilic  
19 acid, and m-hydroxybenzoic acid, with the highest activity towards benzoic acid (Chen et al.  
20 2003). In addition, we noticed that *bsmt1-1* plants have longer petioles than Col-0 and display  
21 leaf epinasty (Online resource 2). Thus, whether any of the methylated metabolites and/or  
22 *bsmt1-1* leaf phenotype influence butterflies for their choice of an oviposition site will need  
23 further investigations.

24 Evidence for the absence of a role for MeSA in response to oviposition and the  
25 observation that egg extract-treatment repelled butterflies implies that other factor(s) may

1 render plants less acceptable for females. First, although egg extracts were applied underneath  
2 *Arabidopsis* leaves, we cannot formally exclude that visual factors informed butterflies about  
3 prior occupancy. Indeed, eggs or egg extract treatment cause chlorosis at the site of deposition  
4 in *Arabidopsis* Col-0 (Bruessow et al. 2010; Reymond 2013). An elegant experiment recently  
5 demonstrated that *P. rapae* butterflies could discriminate green *Arabidopsis* leaves from  
6 variegated green-whitish leaves, obtained after silencing a phytoene desaturase gene (Zheng  
7 et al. 2010). Alternatively, infochemicals from either the egg extract or the plant could be  
8 detected by gravid females. Avenanthramide alkaloids have been identified in eggs of *P.*  
9 *brassicae* and *P. rapae* and were shown to inhibit oviposition when sprayed on cabbage  
10 leaves (Blaakmeer et al. 1994b). However, since cabbage leaves were still repulsive after  
11 removal of *P. brassicae* eggs and avenanthramides were no longer detectable, other plant  
12 chemicals were postulated to deter oviposition although their nature has not been determined  
13 (Blaakmeer et al. 1994a). Glucosinolates (GS) are well-described defense compounds of the  
14 Brassicaceae (Halkier and Gershenzon 2006). Many crucifer specialists use GS as signals for  
15 oviposition, as larvae are able to detoxify them and thus feed unharmed on the plants (Huang  
16 and Renwick 1994; Renwick and Chew 1994; Hopkins et al. 2009). Induction of GS  
17 biosynthesis genes and GS accumulation are triggered by herbivory and are regulated by the  
18 JA pathway in *Arabidopsis* (Schweizer et al. 2013). Since *P. brassicae* eggs were shown to  
19 suppress the expression of JA-dependent defense genes in *Arabidopsis*, including GS-related  
20 genes (Bruessow et al. 2010), an intriguing hypothesis is that GS content might be reduced  
21 after oviposition and therefore this would lower the attractiveness of *Arabidopsis* plants for  
22 further egg laying. In order to test these hypotheses, future studies should aim at measuring  
23 leaf chemical changes or emission of volatiles that follow oviposition in *Arabidopsis* and use  
24 biosynthesis mutants to identify deterring molecules.

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

12 Interestingly, the production/emission of MeSA as well as the expression of *BSMT1*  
13 were also found to be induced after infection with the bacterial pathogen *P. syringae* in  
14 *Arabidopsis*. This effect was due to the presence of coronatine (COR), a bacterial effector that  
15 mimics JA-Ile, which is the bioactive JA (Attaran et al. 2009; Zheng et al. 2012). It would be  
16 interesting to carry out oviposition test with plants inoculated with *P. syringae* to test whether  
17 butterflies avoid infected plants to maximize the survival of their progeny. Use of COR  
18 strains could confirm the role of this effector in oviposition responses.

19 In conclusion, our results suggest that MeSA emission is not responsible for reduced  
20 oviposition by *P. brassicae* on egg-treated *Arabidopsis* plants but that it may rather play a role  
21 during larval feeding to block further oviposition. Whether this is a strategy developed by the  
22 plant to prevent an excess of attackers or by the insect to control food availability for  
23 developing larvae will deserve future studies. A recent meta-analysis offered clear support for  
24 the preference-performance hypothesis which states that female insects evolved to oviposit  
25 more eggs on plants on which their offspring performs best (Gripenberg et al. 2010). This

1 indicates that the avoidance of MeSA-emitting plants by females could be linked to a poorer  
2 performance on such plants.

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15

#### 16 FIGURE LEGENDS

17 **Fig. 1** Dual-choice oviposition tests with *P. brassicae*. Three females and two males *P.*  
18 *brassicae* butterflies were placed in a tent containing two groups of four *Arabidopsis* plants.  
19 The number of eggs laid was assessed after 12 h of continuous light. Plants used were wild-  
20 type (Col-0) unless otherwise specified. Boxplots represent values from six to twenty-five  
21 biological replicates. **a** Number of eggs laid on untreated plants or plants pretreated with *P.*  
22 *brassicae* egg extract (EE) for three days. **b** Number of eggs laid on control plants or plants  
23 placed next to a MeSA dispenser. MeSA (0.15 mg/μl in hexane) was applied to a volatile  
24 dispenser disposed at the center of the pot. Control plants were placed next to a hexane  
25 dispenser. **c** Number of eggs laid on wild-type or *Arabidopsis* OsS6 line that overexpresses  
26 the rice BSMT1 gene. **d** Number of eggs laid on untreated or EE-treated *bsmt1-1* plants. **e**  
27 Number of eggs laid on EE-treated Col-0 or *bsmt1-1* plants. **f** Number of eggs laid on  
28 untreated Col-0 or *bsmt1-1* plants. Oviposition data were analyzed with a *Generalized Linear*  
29 *Model*. Stars indicate a significant difference compared to the control (\*\*\*)  $P < 0.001$ .

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**Fig. 2** Expression of *PR-1* **a** and *BSMT1* **b** after treatment with *P. brassicae* egg extract (EE) for five days or feeding by *P. brassicae* (*P. b.*) for two days. Expression levels were measured by QPCR and are relative to the housekeeping gene *EIF4A1*. Values are mean relative expression  $\pm$  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).

SUPPLEMENTARY MATERIAL

Online resource 1. MeSA analysis of *Arabidopsis* plants with or without *P. brassicae* oviposition.

Online resource 2. Phenotype of Col-0 and *bsmt1-1* plants



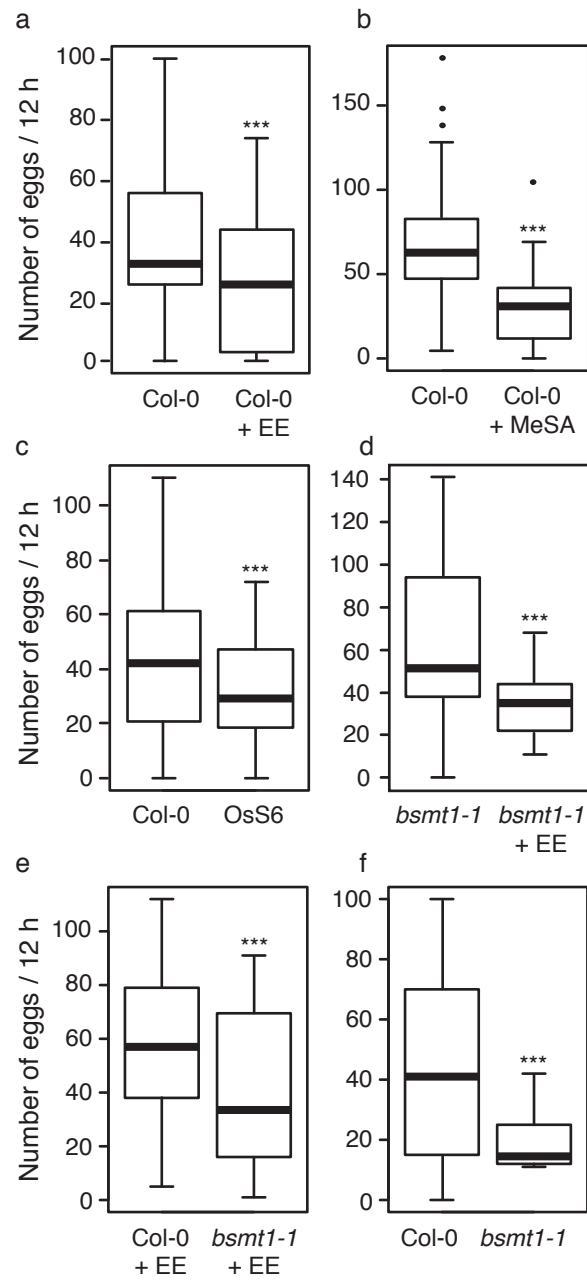


Figure 1

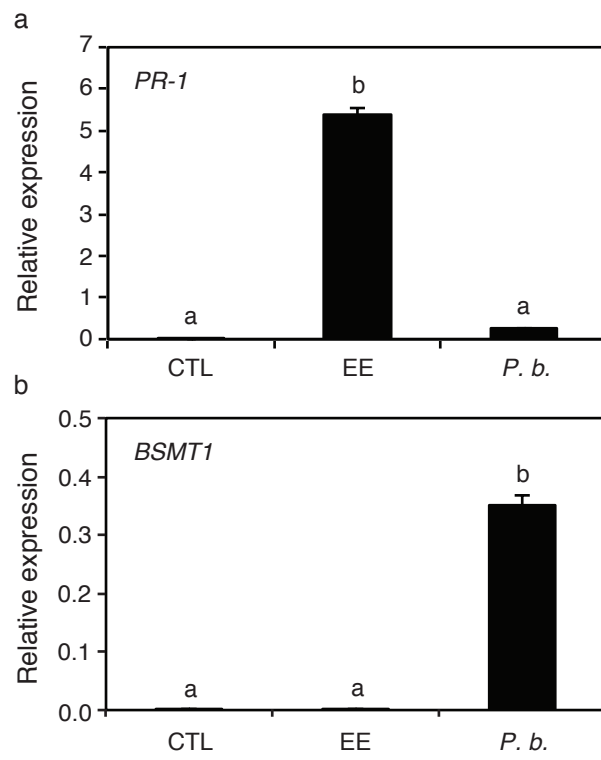
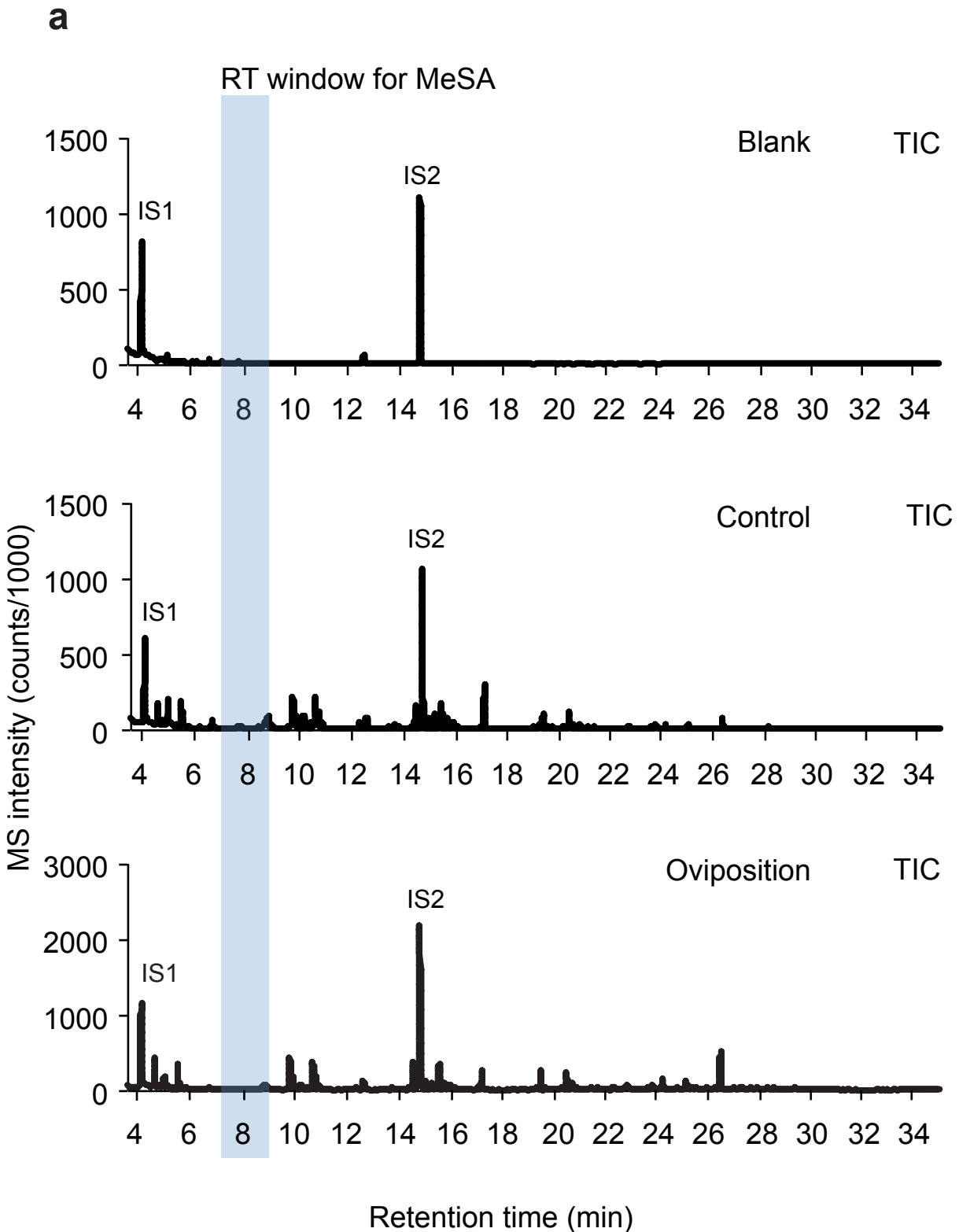
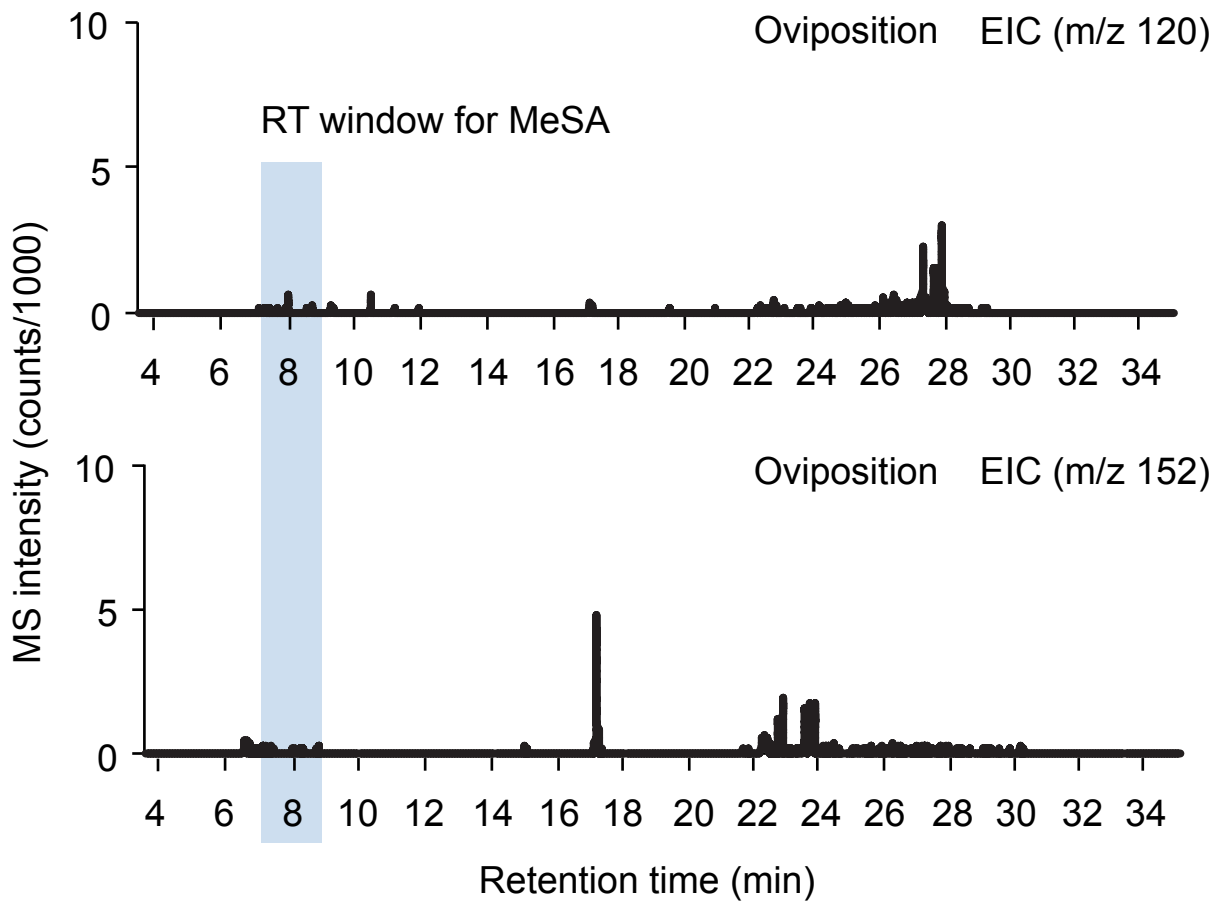
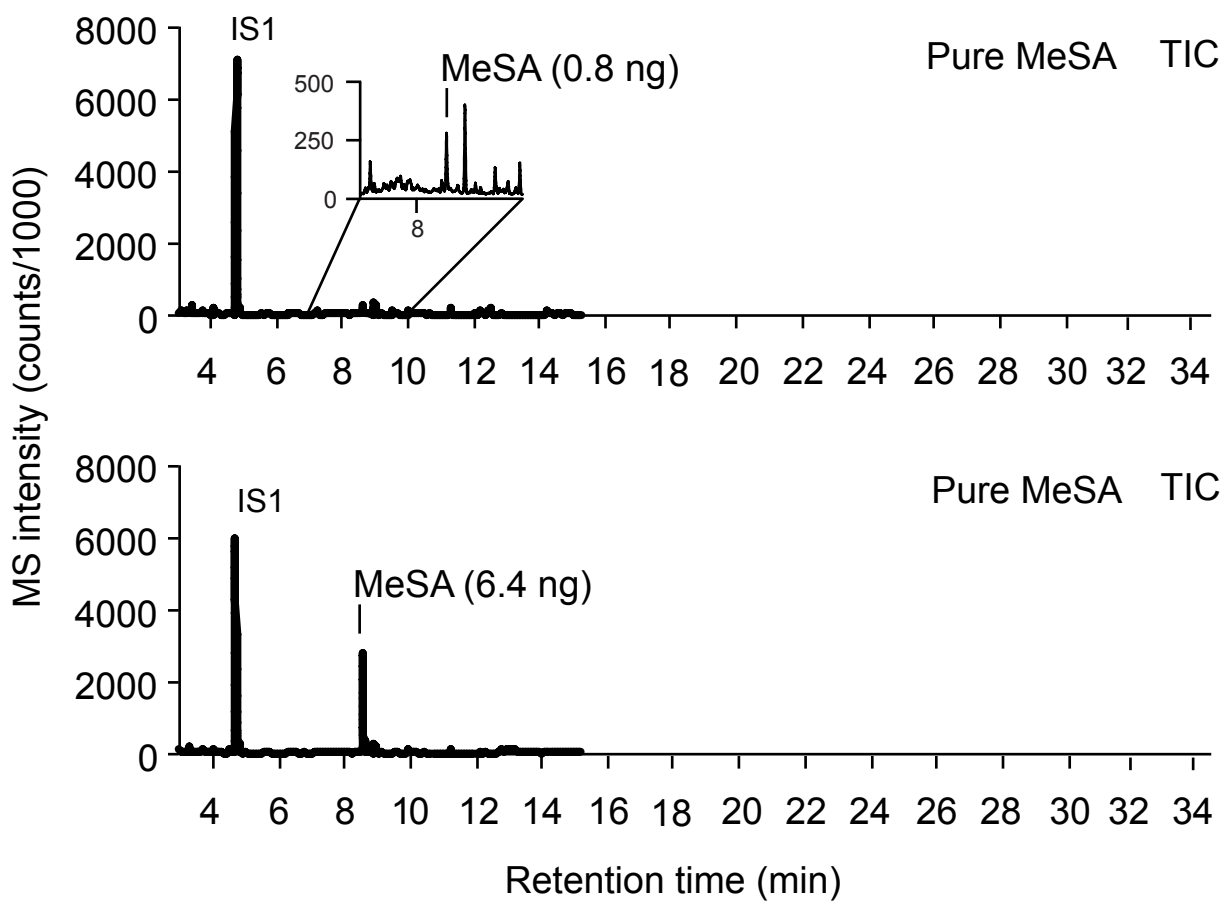


Figure 2

SUPPLEMENTARY MATERIAL

Online resource 1. MeSA analysis of *Arabidopsis* plants with or without *Pieris brassicae* oviposition. Volatiles were collected from 48 h to 72 h after oviposition. **a** Representative total ion chromatograms (TIC) from a blank sample (empty bottle), control plants and oviposited plants. **b** Extracted ion chromatogram (EIC) for ions characteristic of MeSA (mass spectra obtained from NIST05). **c** Total ion chromatogram of pure MeSA (Sigma Aldrich). IS1: internal standard 1 (n-octane); IS2: internal standard 2 (n-nonyl acetate).



**b****c**

Online resource 2. Phenotype of 5-week-old Col-0 and *bsmt1-1* plants.

