

RESEARCH PAPER

# Investigations into plant biochemical wound-response pathways involved in the production of aphid-induced plant volatiles

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## Abstract

Feeding damage to plants by insect herbivores induces the production of plant volatiles, which are attractive to the herbivores natural enemies. Little is understood about the plant biochemical pathways involved in aphid-induced plant volatile production. The aphid parasitoid *Diaeretiella rapae* can detect and respond to aphid-induced volatiles produced by *Arabidopsis thaliana*. When given experience of those volatiles, it can learn those cues and can therefore be used as a novel biosensor to detect them. The pathways involved in aphid-induced volatile production were investigated by comparing the responses of *D. rapae* to volatiles from a number of different transgenic mutants of *A. thaliana*, mutated in their octadecanoid, ethylene or salicylic acid wound-response pathways and also from wild-type plants. Plants were either undamaged or infested by the peach-potato aphid, *Myzus persicae*. It is demonstrated that the octadecanoid pathway and specifically the *COI1* gene are required for aphid-induced volatile production. The presence of salicylic acid is also involved in volatile production. Using this model system, in combination with *A. thaliana* plants with single point gene mutations, has potential for the precise dissection of biochemical pathways involved in the production of aphid-induced volatiles.

Key words: *cev-1*, *coi1-16*, *Col-gl*, *NahG*, *npr-1*, semiochemical, tritrophic interaction, Y-tube olfactometer.

## Introduction

Herbivory by insects can induce biochemical changes in plants that increase their defences against herbivores. These induced defences can either be direct, which cause direct deleterious effects on the herbivore, or indirect, affecting the herbivore via its natural enemies (Dicke and Vet, 1999). Indirect defences are postulated to involve the induction of plant volatiles, which act as host location cues for insect natural enemies. It has been proposed that insect natural enemies foraging for insect hosts/prey may encounter a 'reliability-detectability problem'. This is a hypothesis that states that chemicals produced by herbivore hosts are likely to be highly reliable, taxonomically specific, host location cues for natural enemies, but may have low detectability due to the herbivore host needing to remain inconspicuous. A proposed solution is that interactions between a host herbivore and its food plant could lead to the production of volatiles that may be both reliable and more highly detectable, host location cues (Vet and Dicke, 1992).

Two key plant biochemical pathways have been identified as playing roles in both direct and indirect plant defences; the octadecanoid (OD) pathway, with jasmonic acid (JA) as the key signalling compound, and the salicylic acid (SA) pathway, with SA as the major signalling compound (van Poecke and Dicke, 2002; Smith and Boyko, 2007). In addition, the ethylene (ET) pathway, with ET as the signalling compound, is another important pathway that acts synergistically with JA (Ellis and Turner, 2001; Harfouche *et al.*, 2006; Smith and Boyko,

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Abbreviations: ET, ethylene; JA, jasmonic acid; MeSA, methyl salicylate; OD, octadecanoid; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance; WT, wild-type.

2007). Wound signalling in plants involves branched and interconnecting pathways, allowing 'cross talk' and redundancy when a plant defends itself against herbivory (van Wees *et al.*, 2000; Beckers and Spoel, 2006).

The OD pathway is a key signalling pathway involved in direct defence against insects (McConn *et al.*, 1997). JA and other jasmonates alter a plant's gene transcription, RNA processing and translation, and can induce the production of secondary metabolites and defence proteins (Gundlach *et al.*, 1992; Feys *et al.*, 1994; McConn *et al.*, 1997; Xie *et al.*, 1998; Gols *et al.*, 1999; Brader *et al.*, 2001; Shan *et al.*, 2007). In *Arabidopsis thaliana* Columbia (Brassicaceae) many defence genes are induced in response to mechanical wounding alone (Reymond *et al.*, 2000). The SA pathway is important in plant defence against pathogens (Mauch-man and Métraux, 1998; Heil and Bostock, 2002) and is also activated in response to herbivores associated with low levels of tissue damage, i.e. phloem-feeding insects (Walling, 2000; Moran and Thompson, 2001). Pathogenesis-related (PR) gene RNAs accumulate in response to aphid feeding (Zhu-Salzman *et al.*, 2004; Kempema *et al.*, 2007; Zarate *et al.*, 2007). However, these do not appear to affect herbivore performance deleteriously (Moran and Thompson, 2001).

The signal-transduction pathways involved in indirect defence, against chewing and tissue damaging herbivores such as caterpillars, have begun to be characterized (Hopke *et al.*, 1994; Dicke *et al.*, 1999; van Poecke and Dicke, 2002, 2003, 2004), with the OD pathway reported as being the most important (Dicke *et al.*, 2003). JA accumulates in response to herbivory and is involved in the induction of plant volatiles known to be involved in indirect defences (Thaler *et al.*, 2002; Zhu-Salzman *et al.*, 2004; Pickett *et al.*, 2007). Furthermore, the SA pathway is proposed to have a role in indirect defence against spider mites, since the methyl ester of SA (MeSA) is known to attract predatory mites (Dicke *et al.*, 1990). However, in tomato plants MeSA production is not solely SA-dependent and JA is a key regulator in spider mite-induced MeSA production (Ament *et al.*, 2004).

Phloem-feeding aphids, although able to consume large quantities of phloem sap, do not inflict extensive tissue damage upon the plant (Walling, 2000; Ellis *et al.*, 2002). However, an aphid feeding site can be used for h or even weeks (Tjallingii and Hogen Esch, 1993). Therefore, aphids induce plant biochemical responses distinct from those induced by chewing insects and cell-content feeders. Phloem-feeding herbivores activate pathways more commonly associated with pathogen infection, such as the SA pathway, together with the OD/ET-dependent pathways (Walling, 2000; Moran and Thompson, 2001; Thompson and Goggin, 2006). Characterization of the direct defence pathways against aphids and other phloem-feeding insects is progressing (Moran and Thompson, 2001; Ellis *et al.*, 2002; Hunt *et al.*, 2006; Kempema *et al.*, 2007; Zarate

*et al.*, 2007), as is the analysis of behavioural responses of parasitoids to aphid-induced volatiles (Reed *et al.*, 1995; Du *et al.*, 1996; Powell *et al.*, 1998; Storeck *et al.*, 2000; Blande *et al.*, 2004, 2007; Girling *et al.*, 2006; Pareja *et al.*, 2007; Tentelier and Fauvergue, 2007). However, much less is known about the biochemistry of aphid-induced volatile production by plants.

Wild-type (WT) *A. thaliana*, infested by the peach-potato aphid *Myzus persicae* Sulzer (Homoptera: Aphididae), emit a different volatile blend to that of undamaged plants, due to a biochemical interaction between the plant and the aphid. This blend can be learnt and discriminated by a hymenopteran parasitoid of the aphid, *Diaeretiella rapae* MacIntosh (Hymenoptera: Aphidiidae), and used as a cue in the location of their aphid hosts (Girling *et al.*, 2006). Many parasitoid species are capable of learning precise volatile mixtures and in dual-choice experiments are able to detect extremely subtle differences in volatile compositions and concentrations and show a preference for their learnt source (Reed *et al.*, 1995; Du *et al.*, 1996; Powell *et al.*, 1998; Storeck *et al.*, 2000; Blande *et al.*, 2004; Girling *et al.*, 2006). *Diaeretiella rapae* can therefore be used as a biosensor by giving it a learning experience of a plant and then using it as an extremely sensitive sensor to detect whether volatiles emitted by a plant match those they have learnt. The genetics and biochemistry of both the JA and SA pathways have been extensively researched in *A. thaliana* and mutants altering control at different points of the pathways are available (Cao *et al.*, 1994; Ellis and Turner, 2001, 2002).

It was investigated whether the OD and SA wound response pathways are involved in the production of aphid-induced volatiles, using the *A. thaliana*-*M. persicae*-*D. rapae* tritrophic system, in combination with: (i) two *A. thaliana* OD mutants: the JA insensitive *coil-16* and the JA and ET constitutive *cevl* (Ellis and Turner, 2001, 2002); and (ii) two SA mutants, both of which are impaired in their SA signalling: *NahG*, which is unable to accumulate SA, and the SA insensitive *npr-1* (van Wees and Glazebrook, 2003) (Table 1), as well as WT plants.

**Table 1.** Differential production of, and sensitivity to, jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) by different *A. thaliana* mutants and wild-type (WT) plants, when undamaged or infested by *M. persicae*

Summaries of plant phenotypic expressions reproduced from Ellis and Turner (2001, 2002) and van Wees and Glazebrook (2003).

| <i>A. thaliana</i> mutant | Undamaged <sup>a</sup> |     |     | Infested <sup>a</sup> |      |     |
|---------------------------|------------------------|-----|-----|-----------------------|------|-----|
| WT                        | –                      | –   | –   | JA+                   | SA+  | ET+ |
| <i>Cevl</i>               | JA+                    | –   | ET+ | JA+                   | SA+  | ET+ |
| <i>coil-16</i>            | JA<                    | –   | –   | JA<+                  | SA+  | ET+ |
| <i>NahG</i>               | JA+                    | SA– | –   | JA+                   | SA–  | ET+ |
| <i>npr-1</i>              | JA+                    | SA< | –   | JA+                   | SA<+ | ET+ |

<sup>a</sup> +increased levels; – reduced levels; < insensitive.

Female *D. rapae* were given experience of WT *A. thaliana* plants infested with *M. persicae* and the parasitoid was then used as a biosensor for these plant volatiles by testing their responses and preferences between pairs of different plant volatile sources. In this way the parasitoid was used to identify which plant defence pathways are involved in aphid-induced volatile production by *A. thaliana*. Using this system the following hypotheses were tested. (i) The OD wound response pathway in *A. thaliana*, and specifically the *COI1* gene, are required for the production of *M. persicae*-induced volatiles, which act as host location cues for *D. rapae*. (ii) The SA wound response pathway in *A. thaliana*, and specifically the *NPR1* gene, are required for the production of *M. persicae*-induced volatiles, which act as host location cues for *D. rapae*.

## Materials and methods

### Plants

*Arabidopsis thaliana* Col-*gl* (wild-type [WT]), *cevl* (JA & ET constitutive), *coil-16* (JA insensitive), *NahG* (SA deficient), and *npr-1* (SA insensitive) (Ellis and Turner, 2001, 2002; van Wees and Glazebrook, 2003) seeds were grown separately in a soil mixture consisting of two parts Levington John Innes no. 3 (The Scotts Co. UK Ltd, Ipswich, UK), two parts grit, two parts peat, and one part fine vermiculite at  $23 \pm 2.5$  °C, with a 8/16 h light/dark photoperiod to stimulate vegetative growth and prevent flowering. Seedlings were separated and transferred to individual 5 cm pots approximately 2 weeks after sowing. All plants used were between 6 and 12 weeks old and between 5–7 cm in diameter.

### Insects

*Myzus persicae* were reared on both greenhouse-grown *Brassica rapa* ssp. *pekinensis* (Brassicaceae) and WT *A. thaliana* plants, in separate Perspex cages (50×35×45 cm), with a 16/8 light/dark photoperiod at c. 21 °C.

A population of *D. rapae* were obtained from Rothamsted Research and reared on *M. persicae* grown on *B. pekinensis*. It was not possible to maintain a continuous culture of *D. rapae* on *M. persicae* reared on *A. thaliana*. Therefore, the protocol described by Girling *et al.* (2006) was followed and all wasps were provided with a WT *A. thaliana* plant heavily infested with *M. persicae*, on which they were allowed to mate and gain post-emergence oviposition experience, for 1 d before the trial. In other aphid parasitoid species this is sufficient experience for learning to occur (Du *et al.*, 1996; Powell *et al.*, 1998; Blande *et al.*, 2007).

*Diaeretiella rapae* are solitary koinobiont endoparasitoids and therefore lay one egg inside each host, which hatches into a larva that develops inside the host. The larva spins a cocoon inside the cuticle of the aphid, to form a brown mummy of the aphid. Wasp mummies were collected from *B. rapa* ssp. *pekinensis* plants and adults were allowed to hatch in a blackened chamber with a hole in the lid. Wasps were collected in a Perspex flask, lit from above, which was placed above the hole in the blackened chamber. The flask was changed daily and all wasps that hatched during 1 d were kept in that flask until used. They were fed with aqueous 25% (v/v) honey solution on cotton wool. All bioassay experiments were conducted on female wasps between 1- and 3-d-old (Blande *et al.*, 2007).

### Olfactometer bioassay

Dual-choice tests were carried out using an identical experimental Y-tube and olfactometer apparatus, and the same protocols as described by Girling *et al.* (2006). Experiments were performed using a glass Y-tube olfactometer 2 cm in diameter, with an 18 cm stem and 16 cm arms at a 100° angle. Air was pumped through an activated charcoal filter, through Teflon tubing and divided by a T-junction. The two airflows then passed through two separate flow meters, which regulated the flow rate to 400 ml min<sup>-1</sup>. The air then passed into two 1.0 l flasks with quickfit lids, into which the volatile sources to be tested were placed. From here the air from the flasks flowed into the arms of the olfactometer. The Y-tube was placed in a black-lined, 28×54×42 cm cardboard box, with the front wall and top removed, to reduce any sources of visual bias for the parasitoids. All tests were performed in a constant temperature room at c. 23 °C. The Y-tube was lit from above by two 15 W fluorescent lamps, fitted with a prismatic filter, to ensure a completely even distribution of light. This illuminated the Y-tube with a light intensity of 460 lux. Both parasitoids and volatile sources were placed under the lamp for at least 1 h before any experiments were conducted to allow them to acclimatize to the light level and temperature.

A single female parasitoid was introduced to the Y-tube. Wasps were given 5 min to make a choice, as pilot experiments and previous studies (Du *et al.*, 1996; Girling *et al.*, 2006; Blande *et al.*, 2007) have shown that this is a sufficient time for a response to be elicited. The olfactometer was divided into a number of sections. The first section was the stem, defined as the first 17 cm of the main arm of the Y-tube. The next section was the 'choice zone', the area in which the parasitoid made its 'choice' between the two arms of the olfactometer. It was defined as the first 3 cm of each arm of the olfactometer from the end of the stem. The final sections were the arms of the olfactometer, beyond these first 3 cm. When a parasitoid first entered one arm it was defined as having made a 'choice' between volatile sources. Throughout this paper the term 'attraction' is used to indicate that wasps made a significant choice between odours, i.e. walked towards one volatile source significantly more often than to another. After each individual was tested, the olfactometer was turned over for the next test, to eliminate any directional bias by the parasitoids. Between trials all glassware was washed with acetone and distilled water and then baked at 180 °C to remove any volatiles adhering to the glass.

### Dual-choice experiments

A viable protocol has previously been established for the infestation of test plants, in which plants were infested with 100 *M. persicae* for 3 d prior to testing (Girling *et al.*, 2006). All experiments were performed with wasps given 1 d oviposition experience of *A. thaliana* infested with *M. persicae*.

To confirm the results of previous experiments that *D. rapae* can use volatiles from infested WT *A. thaliana* as host location cues (Girling *et al.*, 2006), the following dual-choice experiment was conducted: (1a) infested (INF) WT versus undamaged (UD) WT.

To test the first hypothesis, that the intact OD wound response pathway in *A. thaliana* is required for the production of *M. persicae*-induced volatiles, which act as host location cues for *D. rapae*, the following dual-choice experiments were conducted: (1b) UD *cevl* versus UD WT; (1c) INF WT versus INF *coil-16*; (1d) INF *coil-16* versus UD *coil-16*; (1e) INF WT versus UD *cevl*; and (1f) INF *cevl* versus UD *cevl* (Table 1).

To test the second hypothesis, that the intact SA wound response pathway in *A. thaliana* is required for the production of *M. persicae*-induced volatiles, which act as host location cues for *D. rapae*, the following dual-choice experiments were conducted: (2a) INF WT versus INF *NahG*; (2b) INF *NahG* versus UD *NahG*; (2c)

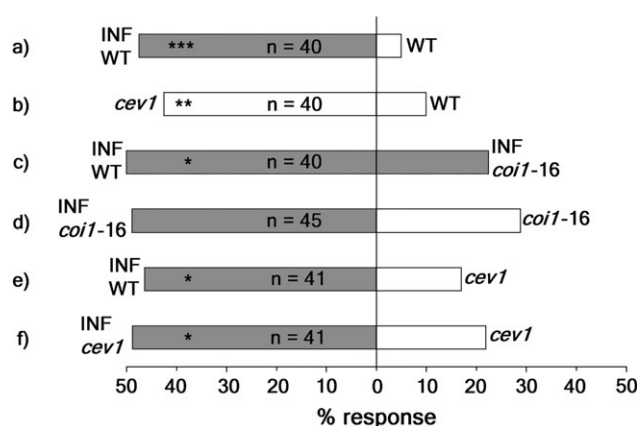
INF WT versus INF *npr-1*; (2d) INF *npr-1* versus UD *npr-1*; and (2e) INF *npr-1* versus INF *NahG*.

Experiments were repeated over several days with 4–7 wasps per pairwise comparison per day, in order to eliminate any daily bias. Data for replicates in each dual-choice experiment were first subjected to a heterogeneity G-test. If not significant they were then pooled and analysed using a pooled G-test for goodness of fit (Sokal and Rohlf, 1995).

## Results

In dual-choice tests, female *D. rapae* showed a significant attraction to volatiles from WT *A. thaliana* infested with *M. persicae*, over those from undamaged WT plants ( $G_1=15.90$ ,  $P < 0.001$ ) (Fig. 1a). This confirms the finding that female *D. rapae*, given oviposition experience on *M. persicae*-infested WT *A. thaliana*, can learn and respond to aphid-induced volatiles from that complex and utilize them as a host location cue (Girling et al., 2006). Because all wasps in this study were given oviposition experience on *M. persicae*-infested WT *A. thaliana* plants, the parasitoids will have learnt the volatile blend of this complex, therefore the parasitoids should show a stronger attraction to blends most similar in composition to those of a WT plant. The wasps' responses were therefore used as an indicator of the 'composition' of the blends produced in each dual-choice test.

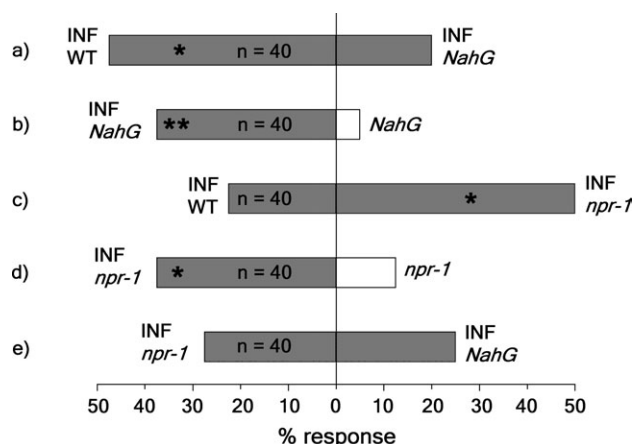
*cev1* plants have constitutive expression of JA and ET (Table 1). Activation of these pathways resulted in female *D. rapae* showing a significant attraction to volatiles from these *cev1* plants, over those from undamaged WT plants ( $G_1=8.66$ ,  $P < 0.01$ ) (Fig. 1b). *coil-16* plants are insensitive to JA but have fully active ET expression (Table 1).



**Fig. 1.** Responses of experienced *D. rapae* in dual-choice tests, in a Y-tube olfactometer, to three different genotypes of *A. thaliana*: Wild-type (WT); jasmonate and ethylene constitutive (*cev1*); and jasmonate insensitive (*coil-16*), either infested with 100 *M. persicae* (INF) (filled squares) or undamaged (UD) (open squares). Horizontal bars indicate the percentage of wasps attracted, in dual-choice tests, to the indicated genotype of *A. thaliana*. Asterisks indicate a significant difference in a choice test: \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$  (G-test). *n* for each treatment is displayed inside the corresponding bars.

Infested *coil-16* plants were significantly less attractive to *D. rapae* than infested WT plants, which possess the full suite of defence responses activated ( $G_1=4.28$ ,  $P < 0.05$ ) (Fig. 1c). Parasitoids were not significantly more attracted to *coil-16* plants, when infested with *M. persicae* compared with undamaged *coil-16* plants ( $G_1=2.34$ ,  $P > 0.05$ ) (Fig. 1d). Parasitoids were significantly attracted to plants, which had their full suite of defence pathways activated over plants which only had their OD and ET pathways activated; both infested WT plants over undamaged *cev1* plants ( $G_1=5.75$ ,  $P < 0.05$ ) (Fig. 1e) and infested *cev1* plants over undamaged *cev1* plants ( $G_1=4.28$ ,  $P < 0.05$ ) (Fig. 1f).

*NahG* plants are unable to accumulate SA (Table 1). Infested WT plants, with a full suite of defence responses, were significantly more attractive to *D. rapae* than infested *NahG* plants ( $G_1=4.61$ ,  $P < 0.05$ ) (Fig. 2a). In addition, volatiles from infested *NahG* were significantly more attractive to *D. rapae* than those from undamaged *NahG* ( $G_1=11.25$ ,  $P < 0.001$ ) (Fig. 2b). *npr-1* plants are insensitive to but accumulate normal levels of SA (Table 1). Parasitoids could distinguish between volatiles from infested WT plants, with a full defence response, and infested *npr-1* plants, showing a significant attraction to infested *npr-1* plants ( $G_1=4.28$ ,  $P < 0.05$ ) (Fig. 2c). Furthermore, *D. rapae* showed a preference for volatiles from infested over undamaged *npr-1* plants ( $G_1=5.23$ ,  $P < 0.05$ ) (Fig. 2d). Parasitoids did not distinguish between volatiles from infested *npr-1* plants, which are insensitive to SA, and those from infested *NahG* plants, which are unable to accumulate SA ( $G_1=0.05$ ,  $P > 0.05$ ) (Fig. 2e). In all cases, where *NahG* was used it attracted



**Fig. 2.** Responses of experienced *D. rapae* in dual-choice tests, in a Y-tube olfactometer, to three different genotypes of *A. thaliana*: Wild-type (WT); salicylic acid-deficient (*NahG*); and salicylic acid-insensitive (*npr-1*), either infested with 100 *M. persicae* (INF) (filled squares) or undamaged (UD) (open squares). Horizontal bars indicate the percentage of wasps attracted, in dual-choice tests, to the indicated genotype of *A. thaliana*. Asterisks indicate a significant difference in a choice test: \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$  (G-test). *n* for each treatment is displayed inside the corresponding bars.



fewer wasps than the test counterpart and where infested *npr-1* was used it attracted greater numbers of wasps than the test counterpart.

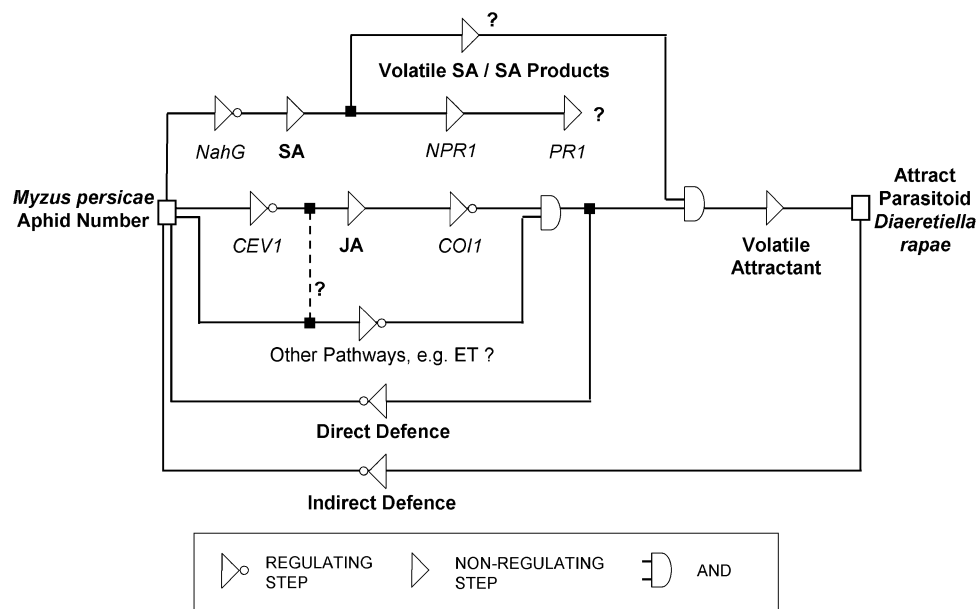
## Discussion

A mechanism is proposed by which *A. thaliana* may defend itself, both directly and indirectly, by utilizing a combination of its OD and SA wound-response pathways (Fig. 3). Aphid infestation induces the expression of these biochemical pathways, which are known to result in direct defence against insects and pathogens (Moran and Thompson, 2001; Ellis *et al.*, 2002; Hunt *et al.*, 2006). Here, some of the biochemical pathways that are involved in indirect defence against aphids are broadly elucidated.

*cev1* *A. thaliana* mutants have constitutive activation of the *CEV1* gene, which regulates an early step in *A. thaliana* defence pathways and stimulates constitutive activation of both the OD and ET pathways (Table 1). *Diaeretiella rapae*, given a learning experience of aphid-infested WT plants, showed a significant preference to volatiles from undamaged *cev1* mutants over undamaged WT plants (Fig. 1b). This indicates that, even without aphid infestation, endogenous activation of the OD and/or ET wound response pathways by the *CEV1* gene results in the emission of volatiles similar to those emitted by an

infested WT plant (Fig. 3). However, because OD and ET are both constitutively activated in the *cev1* mutant, this experiment does not distinguish which of these pathways plays a role in the production of aphid-induced volatiles.

*coil-16* mutants are insensitive to JA and only have a partially-functional OD pathway, due to inactivation of the coronatine-insensitive 1 (*COI1*) gene, but do have a fully functional ET pathway (Table 1). A key function of the OD pathway and the *COI1* gene is to initiate the expression of many secondary metabolite genes, for example, those involved in the production of volatiles (Devoto and Turner, 2003). If the OD pathway, independent of the ET pathway, is important in the production of aphid-induced volatiles, then experienced *D. rapae* should prefer volatiles from infested WT plants than from infested *coil-16* plants. Parasitoids did show this preference (Fig. 1c) and therefore infested *A. thaliana*, without a JA response, do not produce the same aphid-induced volatiles as do infested WT. These results provide support for the first hypothesis, that the OD pathway is required for aphid-induced volatile production in *A. thaliana* and also provides evidence that the *COI1* gene has a regulatory role, within the OD pathway, in the production of these volatiles (Fig. 3). Exogenous treatment of a number of plant species with JA results in the emission of similar volatile blends to those produced by infestation from a number of different herbivore species



**Fig. 3.** Proposed mechanisms and roles of the octadecanoid and salicylic acid plant wound response pathways in the defence of *A. thaliana* against the aphid *M. persicae*. In this proposed system, aphid infestation results in activation of the *CEV1* gene, which regulates a step early in the octadecanoid and ethylene pathways, this results in stimulation of jasmonic acid (JA) and ethylene (ET). The *COI1* gene regulates the response of *A. thaliana* to the JA signal in the octadecanoid pathway and results in the expression of both direct defences against aphids and the production of volatile attractants, which attract the aphid-parasitoid *D. rapae*. It is hypothesized that this acts as an indirect defence. Responses of the ET pathway may also feed into both of these defences. The salicylic acid (SA) pathway is also activated by aphid infestation but does not contribute to direct defence. Transgenic plants with the *NahG* gene inserted break down SA so it cannot accumulate; this removes volatile SA and SA products, which act as volatile attractants, reducing the attractiveness of the volatile attractant signal to parasitoids. Cross talk may also occur in between JA and SA pathways, but it is currently unclear how this influences both direct and indirect defences.

(Dicke *et al.*, 1999; Gols *et al.*, 1999; Thaler, 1999; Birkett *et al.*, 2000; van Poecke and Dicke, 2002). The OD pathway is important for direct defence against *M. persicae* (Ellis *et al.*, 2002) and the current results demonstrate that it is also important for so-called 'indirect defence' against aphids (Fig. 3).

Because *coil-16* mutants are insensitive to JA, if the OD pathway is the only pathway involved in aphid-induced volatile production, then the parasitoid biosensor, which has learnt the full blend of volatiles from infested WT plants, should not show a preference between infested and undamaged *coil-16* plants. In bioassays, *D. rapae* did not show a preference for volatiles from infested and undamaged *coil-16* (Fig. 1d). However, there was some attraction, though below the significance level, to the infested plant (Fig. 1d). Furthermore, *D. rapae* showed a significant preference for volatiles from infested WT plants, with all defence pathways available for activation, over undamaged *cevl*, which only have their JA and ET pathways activated, (Fig. 1e) and a significant preference for volatiles from infested *cevl*, which have all the defence pathways activated, over undamaged *cevl* (Fig. 1f). This indicates that aphid-infestation of *A. thaliana* is required to produce the full blend of induced volatiles attractive to the parasitoid biosensor and therefore suggests that pathways other than OD and ET may be involved in the production of aphid-induced volatiles. Similarly, the parasitoid wasp *Cotesia rubecula* displayed a preference for volatiles emitted from *A. thaliana* infested with *Pieris rapae* over those from plants sprayed with JA (van Poecke and Dicke, 2002).

*NahG* is a transgenic plant transformed to constitutively express the *Pseudomonas putida NahG* gene that encodes the expression of salicylate hydroxylase, an enzyme that converts salicylic acid into inactive catechol, and therefore *NahG* is unable to accumulate SA (van Wees and Glazebrook, 2003) (Table 1). Parasitoids significantly preferred infested WT plants over infested *NahG* plants (Fig. 2a), providing support for the third hypothesis and suggesting that the presence of SA or SA derived volatiles are required for aphid-induced volatile production (Fig. 3). Similarly, parasitic wasps of the caterpillar, *P. rapae*, are less attracted to caterpillar-infested *NahG* than to caterpillar-infested WT plants (van Poecke and Dicke, 2002). Aphids activate SA signalling pathways more strongly than they elicit JA-sensitive signalling (Thompson and Goggin, 2006). The activation of the SA pathway has no effect on aphid reproduction by direct defence (Moran and Thompson, 2001), but it has been suggested that its expression may be required for indirect defence (van Poecke and Dicke, 2003). Methyl salicylate, which is often released upon aphid-feeding (Walling, 2000) and is known to attract certain natural enemies (Dicke *et al.*, 1990), may not be produced or emitted in *NahG* plants, due to low levels of SA (van Poecke and Dicke, 2002).

However, it is also a possibility, although unlikely, that catechol, produced as a result of the degradation of SA by the *NahG* gene, is acting as a repellent upon *D. rapae* (van Poecke and Dicke, 2002).

Parasitoids were significantly more attracted to volatiles from infested *NahG* than uninfested *NahG* (Fig. 2b). Similarly, caterpillar-infested *NahG* were more attractive to caterpillar parasitoids than undamaged *NahG* (van Poecke and Dicke, 2002). The attraction to infested *NahG* is likely to be due to volatiles being produced independently of SA, associated with other biochemical pathways. Undamaged *NahG* accumulate 25 times more JA than WT and exhibits enhanced expression of JA-responsive defence genes (Spoel *et al.*, 2003), probably due to the antagonistic relationship between SA and JA (i.e. when SA is at a low concentration within a plant JA is at high levels and vice versa). Therefore, it is likely that *NahG* will emit elevated levels of JA-induced volatiles following herbivory.

*npr-1* mutants can accumulate but are effectively insensitive to SA (Table 1). *npr-1* have a defective *NPR1* gene, the normal function of which is to transduce the SA signal leading to the synthesis of defence-related genes (Cao *et al.*, 1997; van Poecke and Dicke, 2003), as a result *npr-1* accumulate normal SA levels and elevated JA levels (Clarke *et al.*, 2000; Spoel *et al.*, 2003) and exhibit enhanced JA-responsive gene expression (van Wees and Glazebrook, 2003). Infested *npr-1* were more attractive to *D. rapae* than both infested WT (Fig. 2c) and undamaged *npr-1* (Fig. 2d). The enhanced JA expression in *npr-1* is likely to result in a greater production of volatiles, which may have increased the attraction of *D. rapae* to *npr-1* in comparison to WT (Fig. 3). Alternately *npr-1* plants may produce a different volatile, which generates a large innate response by the parasitoid biosensor.

Because of the enhanced JA expression in *npr-1* it was not possible to distinguish the role of SA sensitivity in aphid-induced volatile production by using this plant. Therefore, it can only be concluded, from the *NahG* results, that the presence of SA is required for aphid-induced volatile production but not whether SA is merely emitted as a volatile attractant or whether it is acting as a signalling chemical in the SA pathway resulting in the production of other volatiles. The current study utilizes mutants in which the mutations result in very broad changes to their wound defence pathways. 'Cross talk' occurs between wound response pathways and different pathways can suppress or enhance activation of other pathways (Turner *et al.*, 2002; Beckers and Spoel, 2006; Zarate *et al.*, 2007). Therefore, a problem with the mutants used in this study, which have whole pathways either deactivated or constitutively activated, is that there are likely to be large, unknown effects on product formation. Whilst it is possible to identify what effect broad pathway changes have on volatile production, and

therefore resultant attraction of the biosensor, it is sometimes difficult to identify conclusively whether the resultant behavioural responses of the biosensor are caused by the deactivation/activation of one pathway or by the effects that this change has on other pathways. However, other mutants with single gene point mutations are available, which could be utilized in future studies to overcome these problems. These types of mutants have been used to investigate direct defence against aphids (Hunt *et al.*, 2006). Furthermore, using the methodologies set out in this paper, these mutants could be used to identify specific genes or groups of genes, which are involved in the production of aphid-induced volatiles by *A. thaliana*.

*npr-1* and *NahG* are similar in that they both do not mount a SAR or express *PR* genes, and both have elevated JA and JA-responsive gene expression. The presence of SA in *npr-1* may be responsible for infested *npr-1* being more attractive to *D. rapae* than infested WT plants (Fig. 2c), whereas infested *NahG* were less attractive than infested WT (Fig. 2a). Given the differences in attraction of *D. rapae* between infested *npr-1* and *NahG* over infested WT, it is unclear why there was no significant difference in attraction to infested *npr-1* over infested *NahG* (Fig. 2e).

The parasitoid biosensor described in this paper has distinct advantages over most traditional analytical equipment, as a tool for investigating plant signalling, due to the sensitivity of the parasitoids responses. Using parasitoids also allows investigation of the adaptive functions of the volatile cues, because they are known to be important in tritrophic interactions. This study clearly demonstrates that the *A. thaliana*–*M. persicae*–*D. rapae* tritrophic system could be used in combination with more traditional techniques, such as plant molecular studies and quantitative analysis of volatile components, for the precise dissection of biochemical wound-response pathways involved in aphid-induced volatile production within *A. thaliana* (van Poecke and Dicke, 2004). Furthermore, this system could lead to the development of novel methods for aphid control, for example, by using similar methods to those developed for the genetic modification of *A. thaliana* to reduce caterpillar damage by making plants more attractive to their parasitoids (Shiojiri *et al.*, 2006).

In summary, this study indicates roles for the OD pathway, and specifically the *COII* gene, and for SA, or SA-derived volatiles, in the production of aphid-induced volatiles by *A. thaliana* (Fig. 3). Without the OD pathway and *COII* expression, *A. thaliana* were unable to mount a detectable signal to attract parasitoids. Furthermore, the results are compatible with the possibility that other pathways, such as the ET pathway, may also be implicated in the production of the full volatile blend.

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