Induced parasitoid attraction by *Arabidopsis thaliana*: involvement of the octadecanoid and the salicylic acid pathway

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Abstract

Plants can use indirect defence mechanisms to protect themselves against herbivorous insects. An example of such an indirect defence mechanism is the emission of volatiles by plants induced by herbivore feeding. These volatiles can attract the natural enemies of these herbivores, for example, parasitoid wasps. Here, it is shown that the octadecanoid and the salicylic acid pathways are involved in the induced attraction of the parasitoid wasp *Cotesia rubecula* by *Arabidopsis thaliana* infested with the herbivore *Pieris rapae*. Besides exogenous application of jasmonic acid or salicylic acid, use is also made of transgenic *Arabidopsis* that do not show induced jasmonic acid levels after wounding (S-12) and transgenic *Arabidopsis* that do not accumulate salicylic acid (NahG). Treatment of *Arabidopsis* with jasmonic acid resulted in an increased attraction of parasitoid wasps compared with untreated plants, whereas treatment with salicylic acid did not. Transgenic plants impaired in the octadecanoid or the salicylic acid pathway were less attractive than wild-type plants.

Key words: *Cotesia rubecula*, jasmonic acid, *Pieris rapae*, plant volatiles, tritrophic interactions.

Introduction

Plants employ both constitutive and induced defences against pathogens and herbivorous arthropods. Induced defences increase upon attack by pathogens or herbivores. Such inducibility of defences requires information processing in the plant. Signal transduction pathways involved in this information processing have been studied for several years. Two pathways appear to be especially important: the octadecanoid pathway, with the plant hormone jasmonic acid (JA) as one of the key compounds (Karban and Baldwin, 1997), and the salicylic acid pathway, with the plant hormone salicylic acid (SA) as a key compound (Dempsey *et al*., 1999). The importance of these pathways in inducible defences has been demonstrated in many plant species (Dicke and van Poecke, 2002).

The salicylic acid pathway plays an important role in the protection of plants against many pathogen species (Dempsey *et al*., 1999). Although herbivory can lead to an increase of endogenous SA levels or activation of SA-inducible genes, this does not seem to have a negative effect on herbivore performance (Bi *et al*., 1997; Moran and Thompson, 2001). The octadecanoid pathway is involved in the protection against herbivorous insect and mite species (Karban and Baldwin, 1997), but also against microbial pathogens (Thomma *et al*., 1998). The pathways also interact, with SA having an inhibitory effect on the octadecanoid pathway (Peña-Cortés *et al*., 1993) and vice versa (Niki *et al*., 1998). Conversely, JA and SA are also reported to act synergistically (Xu *et al*., 1994).

Most of the studies mentioned above addressed the direct defence of plants against their enemies. However, plants can also defend themselves indirectly by enlisting the enemies of their enemies: carnivores, such as predators of parasitoids (Dicke, 1999a, b). As in direct defences, indirect defences can be constitutively present or inducible (Dicke and van Poecke, 2002). The emission of volatiles upon herbivore attack has received most attention (Dicke, 1999b; Paré and Tumlinson, 1999). These volatiles can attract carnivores, thus reducing the negative impact of the herbivores on the plant and increasing plant fitness (van
The volatile blend emitted upon herbivory is often complex. It can be specific for the inducing herbivore and carnivores can exploit this when locating their herbivorous host or prey species (Dicke, 1999a). In the past few years, several studies have indicated that both the octadecanoid and the salicylic acid pathway play an important role in the attraction of carnivores (Dicke and van Poecke, 2002).

In short, both pathways appear to be involved in direct defences against both pathogens and herbivores and in the indirect defences against herbivores. This may lead to situations where the induction of plant defence against a pathogen reduces or increases resistance of the plant against a herbivore, and vice versa (Felton et al., 1999; Stout et al., 1999). How do plants cope with such complex situations where interactions with pathogens, herbivores and carnivores need to be considered?

To be able to answer this question one needs to study these interactions in the same plant species. The value of the model plant Arabidopsis thaliana in unravelling signal transduction pathways in direct defences against pathogens has been demonstrated in numerous publications (Thomma et al., 1998; Pieterse and Loon, 1999). Direct defences against herbivores have also been studied using Arabidopsis (Mauricio and Rausher, 1997; McConn et al., 1997). Recently, it has been demonstrated that Arabidopsis can be used for studying indirect plant defences: Arabidopsis attracts parasitoid wasps (Cotesia rubecula) upon infestation with Pieris rapae caterpillars (van Poecke et al., 2001). Parasitization of P. rapae by C. rubecula results in an increased seed production by P. rapae-infested Arabidopsis plants compared to Arabidopsis plants infested by unparasitized P. rapae (van Loon et al., 2000). Arabidopsis, therefore, is an excellent candidate to study the interaction between direct and indirect plant defences against herbivores and pathogens.

To do so, a better understanding of signal transduction pathways involved in indirect defences is necessary. Until now, studies on the role of the octadecanoid and the salicylic acid pathway in indirect defence used the exogenous application of JA or JA-like compounds (Dicke and van Poecke, 2002), JA inducers and JA inhibitors (Piel et al., 1997) or methyl salicylate (Ozawa et al., 2000). In this paper, transgenic Arabidopsis plants that do not show wound-induced elevation of JA (S-12; Bell et al., 1995) and transgenic Arabidopsis plants that do not accumulate SA (NahG; Delaney et al., 1994) are used, in addition to exogenous application of JA and SA. The present study’s experiments demonstrate that both the octadecanoid and the salicylic acid pathway are involved in the attraction of the parasitoid wasp C. rubecula to P. rapae-infested Arabidopsis.

**Materials and methods**

**Plants**

*Arabidopsis thaliana* ecotype Columbia (Col-0), the transgenic NahG line (*Arabidopsis thaliana* ecotype Col-0 transformed with the bacterial NahG gene; Delaney et al., 1994) and the transgenic S-12 line (*Arabidopsis thaliana* ecotype Col-0 transformed with the antisense Arabidopsis Lox2 gene; Bell et al., 1995) were grown from seed in a greenhouse (20–30 °C, 50–70% RH, L8:D16). A few days prior to the experiments, 8–10-week-old plants were transferred to a climate room (23±1 °C, 50–70% RH, 10 klx, L8:D16). All plants used were in the vegetative state.

**Insects**

Pieris rapae was reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Icarus) in a climate room (21±1 °C, 50–70% RH, L16:D8).

The parasitoid Cotesia rubecula was reared on *P. rapae* larvae feeding on Brussels sprouts plants, under greenhouse conditions (25±5 °C, 50–70% RH, L16:D8). For bioassays, *C. rubecula* pupae were collected and transferred to a gauze cage in a climate room (23±1 °C, 50–70% RH, L16:D8). The emerging wasps were provided with water and honey. These adult wasps, that did not have contact with plant material or caterpillars (no oviposition experience), are referred to as ‘naive’ wasps.

**Plant treatments**

Caterpillar-infested plants were obtained by placing first instar *P. rapae* larvae on each plant (for the numbers of caterpillars used see the legends of the corresponding figures). The larvae had fed for 24 h on the plants before these plants were used in experiments.

Undamaged (control) plants did not receive any treatment, but were of the same age and size as the treated plants and were transferred to the climate room at the same time.

Wild-type (Col-0) plants treated with jasmonic acid were sprayed in a group of four with in total 5 ml of a 1.0 mM (±)-jasmonic acid (Sigma-Aldrich) solution, 24 h before an experiment.

Wild-type (Col-0) plants treated with salicylic acid were sprayed in a group of eight with in total 10 ml of a 5.0 mM salicylic acid (Sigma-Aldrich) solution, 24 h before an experiment.

**Bioassay**

Parasitoid two-choice flight experiments were conducted in a wind tunnel set-up (25±5 °C, 50–70% RH, 0.7 klx) described by Geervelt et al. (1994), that was modified according to van Poecke et al. (2001), except for the JA bioassay (see below).

For the flight experiments, two odour sources were placed at the upwind end of the wind tunnel. Each odour source consisted of eight *Arabidopsis* plants, all having received the same treatment. One day before a bioassay, 4–7-d-old, naive *C. rubecula* wasps were sexed and the males removed. Just prior to the bioassay, an individual female wasp was placed on a microscope slide with one leaf from a *P. rapae*-damaged wild-type Arabidopsis, from which the caterpillars had been removed. The slide, with wasp and leaf, was transported to the middle of the release cylinder in the wind tunnel, which was 60 cm downwind of the odour sources.

For the JA bioassay, each odour source consisted of four instead of eight *Arabidopsis* plants. The female wasps were released from a glass vial (diameter 1.5 cm, length 5 cm), after a 10 min experience with three leaves from a caterpillar-damaged wild-type *Arabidopsis*, from which the caterpillars had been removed.

The flight behaviour of individual wasps was observed. Only flights that resulted in the first landing on one of the odour sources were recorded as ‘choice’. Landings on other parts of the wind tunnel besides the release cylinder or odour sources were recorded as
Fig. 1. Response of C. rubecula to jasmonic acid-treated wild-type Arabidopsis compared to P. rapae-infested plants. In a two-choice set-up the response of naive C. rubecula females was tested to undamaged wild-type plants (C-wt), wild-type plants infested with 15 first instar P. rapae caterpillars 24 h before an experiment (Pr-wt), and wild-type plants treated with a 1 mM jasmonic acid solution 24 h before an experiment (JA-wt). The percentage of in total 55 wasps (this total includes the wasps not making a choice) per combination, choosing one odour source or the other, is shown. Asterisks indicate a significant difference within a choice test: ** * P<0.01, * P<0.05 (χ²-test).

‘no choice’. If the wasp remained in/on the release cylinder for longer than 15 min this was also recorded as ‘no choice’. After a ‘choice’ or ‘no choice’ the wasp was discarded.

In all experiments, three odour sources were compared in three pairwise comparisons on each experimental day. Per pairwise comparison of odour sources, positions were changed from left to right and vice versa, to compensate for unforeseen asymmetric effects. These experiments were repeated on several days with 4–10 wasps per pairwise comparison per day.

Choices between two odour sources in the bioassays were statistically analysed using a chi-square test.

Damage analysis
To compare the amount of tissue eaten by P. rapae from wild-type, NahG and S-12 plants, all leaves of a P. rapae-infested plant were cut off and taped on a white paper sheet. This sheet was photocopied. Using these photocopies, both the perimeter and the area of damage per leaf were traced on a transparent sheet using a coloured permanent marker. The transparent sheet was scanned using a flatbed scanner (Hewlett Packard ScanJet 3300 C). Per plant, the number of coloured pixels was counted with a Gif-View computer program (kindly provided by Roland van Zoest, Alterra, Wageningen, the Netherlands).

To compare P. rapae-infested wild-type and NahG plants, 10 first instar larvae per plant were used. After 24 h the leaves of these plants were cut off, taped on paper, and photocopied. In total 27 plants per treatment were analysed.

The amount of tissue eaten by P. rapae on S-12 and wild-type plants was compared using the P. rapae-infested wild-type and S-12 plants from the wind tunnel bioassay. Immediately after the bioassay, the leaves of these plants were cut off, taped on paper, and photocopied. In total 40 infested wild-type and 40 infested S-12 plants were analysed.

Both the perimeter and the area of damage were compared using a two-tailed Wilcoxon-Mann-Whitney ranking test.

Results
Influence of the octadecanoid pathway on parasitoid behaviour
To test the effect of JA treatment on the attractiveness of Arabidopsis plants towards parasitoid wasps, wild-type Arabidopsis plants were sprayed with a 1 mM JA solution. JA-treated wild-type Arabidopsis attracted more C. rubecula parasitoid wasps than untreated plants (P=0.046; Fig. 1). Similarly, wild-type plants infested with P. rapae caterpillars attracted more C. rubecula wasps than uninfested plants (P=0.041; Fig. 1), as has been demonstrated previously (van Poecke et al., 2001). When JA-treated plants were offered against P. rapae-infested plants, the parasitoids distinguished between the odour blends, preferring the P. rapae-infested plants (P=0.008; Fig. 1).

Besides exogenous spraying of JA, the influence of the octadecanoid pathway on parasitoid behaviour was also studied by using transgenic S-12 plants. These S-12 plants do not show a wound-induced JA accumulation because of co-suppression of the lox2 gene (Bell et al., 1995). S-12 plants infested with P. rapae caterpillars attracted less C. rubecula females than wild-type plants infested with P. rapae caterpillars (P=0.0016; Fig. 2). Yet, P. rapae-infested S-12 plants attracted more wasps than undamaged S-12 plants (P=0.0001; Fig. 2). Similarly, P. rapae-infested wild-type plants attracted more wasps than undamaged S-12 plants (P=4x10⁻⁷; Fig. 2).

Influence of the octadecanoid pathway on herbivore behaviour
Possibly, the differences in attraction of wasps by caterpillar-infested S-12 and wild-type plants could be explained by differences in the amount of feeding by P. rapae caterpillars on these plants. To test this, the damage inflicted by five first instar caterpillars was measured after 24 h of feeding for both S-12 and wild-type plants. The damage inflicted by the caterpillars on S-12 plants did not differ from the damage inflicted on wild-type plants, when the area of the removed leaf tissue was compared (P=0.41; Fig. 3A) or when the perimeter of the damaged area was compared (P=0.052; Fig. 3A).
Influence of the salicylic acid pathway on parasitoid behaviour

Although SA treatment of plants induces many direct defence-related processes (Dempsey et al., 1999), as far as is known, the effect of SA application on indirect defences has not been studied so far. Therefore, the effect of spraying wild-type Arabidopsis plants with a 5 mM salicylic acid solution on the attraction of C. rubecula was tested. Treatment with SA did not influence the attraction of parasitoids (Fig. 4). There was no difference when plants treated with SA were tested against undamaged wild-type plants (P=0.59). P. rapae-infested wild-type plants were clearly preferred over both undamaged (P=2×10⁻⁵) and SA-treated plants (P=0.0011).

To study the role of the SA pathway in indirect defences further, NahG plants were used that are unable to accumulate salicylic acid (Delaney et al., 1994). These NahG plants were less attractive when infested by P. rapae, compared to infested wild-type plants (P=0.020; Fig. 5). P. rapae-infested NahG plants were preferred over undamaged NahG plants (P=2×10⁻³; Fig. 5). Similarly, P. rapae-infested wild-type plants were preferred over undamaged NahG plants (P=3×10⁻⁶; Fig. 5).

Influence of salicylic acid on herbivore behaviour

Just as for the S-12 plants, it was tested whether the differences in attraction of wasps by infested NahG and wild-type plants could be explained by differences in the amount of feeding by P. rapae caterpillars on these plants. The damage inflicted by 10 first instar P. rapae larvae on NahG plants did not differ from the damage inflicted by an equal number of first instar larvae on wild-type plants, when measured using the area of the removed leaf tissue (P=0.31; Fig. 3B) or when measured using the perimeter of the damaged area (P=0.44; Fig. 3B).

Discussion

The role of the octadecanoid pathway in direct plant defences against herbivores is well established. Mechanical wounding results in an increase of endogenous jasmonic acid levels, as has been demonstrated in several plant species including Arabidopsis (Peña-Cortés et al., 1993; Bell et al., 1995; Reymond et al., 2000). These increased levels of JA induce the expression of many genes involved in plant defence (Reinbothe et al., 1994). Herbivory can also induce the production of JA, as has been shown in bean (Blechert et al., 1995). Moreover,
herbivory leads to higher JA levels than mechanical damage in tobacco (McCloud and Baldwin, 1997). Besides JA other members of the octadecanoid pathway, for example, the JA precursor 12-oxo-phytodienoic acid (OPDA; Reymond et al., 2000), also accumulate after wounding and these can mediate defences as well (Stintzi et al., 2001).

Evidence is accumulating that the octadecanoid pathway is also involved in indirect defences. Herbivore induced volatiles are not just passively released, but are synthesized de novo (Paré and Tumlinson, 1999). Such inducibility of volatile production requires information transport, for example, by signal molecules. Exogenous application of JA results in emission of a volatile blend that is similar to that emitted upon herbivore attack (Dicke et al., 1999). The same is true for the exogenous application of methyljasmonate (Kessler and Baldwin, 2001), and JA-inducing compounds like cellulysin, a crude cellulase extract from Trichoderma viride (Piel et al., 1997) and certain fatty acid-amino acid conjugates (Halitschke et al., 2001). Moreover, inhibition of the JA pathway blocked the emission of volatiles induced by cellulysin (Piel et al., 1997). The volatile blends of plants treated with JA attract carnivores in the laboratory (Dicke et al., 1999; this paper) and in the field (Thaler, 1999).

Additionally, treatment of lima bean with OPDA induced the emission of volatiles that were not induced by JA, but were induced by spider mite feeding (Dicke et al., 1999; Koch et al., 1999). These OPDA-induced volatiles attract predatory mites (Dicke and van Poecke, 2002).

These data clearly demonstrate the role of the octadecanoid pathway in the attraction of parasitoid wasps by Arabidopsis. Herbivore-infested transgenic S-12 plants, that show a strongly reduced expression of lox2 and do not show wound-induced accumulation of JA, were less attractive to C. rubecula parasitoids than herbivore-infested wild-type Arabidopsis (Fig. 2). This is not due to reduced feeding by P. rapae, as the caterpillars remove equal amounts of tissue from transgenic S-12 plants and wild-type plants (Fig. 3A). The reduced attraction of parasitoids by herbivore-infested S-12 plants can be explained in two ways.

1. The S-12 plants have an altered production of signalling compounds like JA and this has an effect on volatile-emissions after herbivory. In this case, the octadecanoid pathway is involved as a signal-transduction pathway.

2. The reduced expression of lox2 has a direct effect on the production of volatiles. In this case the octadecanoid pathway is involved as a production pathway of volatiles. The products of lipoxygenase are not only used to produce plant hormones like jasmonic acid and OPDA, but also to produce green leaf volatiles, like hexanol and (Z)-3-hexen-1-ol (Gardner, 1995), which are present in the volatile blend from herbivore-infested Arabidopsis (van Poecke et al., 2001). As the S-12 plants are lipoxygenase2 cosuppressed, it is possible that the production of these green leaf volatiles is influenced directly. Females of the parasitoid wasps Microplitis croceipes are attracted by green leaf volatiles (Whitman and Eller, 1990) and the chemoreceptors on the antennae of both C. rubecula and Cotesia glomerata females respond to them (H Smid, unpublished results). Changes in green leaf volatile production could therefore influence parasitoid attraction.

However, JA application on wild-type Arabidopsis results in attraction of the parasitoids (Fig. 1), demonstrating a signalling role of JA in the production of parasitoid attractants. Thus, the octadecanoid pathway is involved as a signal-transduction route in herbivore-induced attraction of parasitoids by Arabidopsis, but possibly also as a production route of parasitoid-attracting volatiles. The results with the S-12 plants also demonstrate that the herbivore-induced attraction of parasitoids by Arabidopsis is not totally dependent on wound-induced JA accumulation: although caterpillar-infested S-12 plants were less attractive than caterpillar-infested wild-type plants, they were still much more attractive than undamaged plants.

The role of salicylic acid in direct defence against herbivores is less clear. Aphid feeding results in the up-regulation of SA-responsive genes in Arabidopsis, although this did not seem to affect aphid performance (Moran and Thompson, 2001). Similarly, feeding by the cotton bollworm (Helicoverpa zea) resulted in elevated SA levels in cotton, but did not affect the performance of H. zea (Bi et al., 1997). Spraying BTH, a SA analogue, actually reduced the resistance of tomato against H. zea (Stout et al., 1999). Transgenic tobacco plants with higher SA levels showed reduced resistance against H. zea whereas plants with lower SA levels show increased resistance (Felton et al., 1999).

A direct role for SA in indirect defences is more evident. A volatile analogue of SA, methyl salicylate (MeSA), is a common component of the blend emitted by herbivore-infested plants (Dicke and van Poecke, 2002), including caterpillar-infested Arabidopsis (van Poecke et al., 2001). As a single compound it can attract predatory mites (Dicke et al., 1990). The chemoreceptors of the parasitoid wasps Cotesia rubecula and C. glomerata (H Smid, unpublished results) and of the predatory mite Phytoseiulus persimilis (De Bruyne et al., 1991) are sensitive to MeSA. In addition, data from Ozawa et al. (2000) suggest a signalling role for SA in indirect defences: treatment of lima bean plants with MeSA induced the emission of several volatiles. Treatment of lima bean with both JA and MeSA induced a volatile blend that closely resembles the blend of spider mite-infested lima bean, whereas treatment with JA only resulted in a blend that was more similar to that of caterpillar-infested plants.

Although application of a relatively high dose of SA on undamaged wild-type Arabidopsis did not increase the
attractiveness (Fig. 4), results obtained using caterpillar-infested transgenic NahG plants (that do not accumulate SA) suggest that SA is involved in parasitoid attraction. Caterpillar-infested NahG plants were less attractive to C. rubecula females than caterpillar-infested wild-type plants (Fig. 5). This is not due to reduced feeding by the P. rapae, as the caterpillars remove equal amounts of tissue from transgenic NahG plants and wild-type plants (Fig. 3B). The reduced attractiveness of caterpillar-infested NahG plants can be explained in several ways. (1) As the concentration of SA in NahG plants is very low, it is likely that this has an effect on the production and emission of MeSA. (2) Salicylic acid is involved in the signalling pathway of herbivore-induced volatile production. (3) The low level of SA in NahG plants has an effect on the jasmonic acid pathway. However, this does not explain the reduced attractiveness of caterpillar-infested NahG plants. As SA has an inhibitory effect on JA production, one would expect that NahG plants have elevated JA levels. And application of JA results in an increased attraction of parasitoid wasps (Fig. 1)! (4) A repellent effect of the degradation products of SA in NahG plants, like catechol, cannot be excluded. However, preliminary data on the volatile blends emitted by caterpillar-infested NahG plants do not support this.

This study clearly demonstrates that Arabidopsis is an excellent tool with which to study the signalling pathways involved in herbivory-induced parasitoid attraction. To get a better understanding of the role of the octadecanoid pathway, mutant plants are available that are not altered in the production of JA, but in the signal transduction pathways downstream of JA. Similarly, mutant plants that are altered in the signalling pathway downstream of SA will be helpful in determining whether the role of SA in Arabidopsis is limited to the provision of a precursor for MeSA, or whether it functions as a signalling compound involved in the herbivory-induced attraction of parasitoids.

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