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Species-specific responses of pine sesquiterpene synthases to sawfly oviposition

Diana Köpke [a,b], Ivo Beyaert [b], Jonathan Gershenzon [a], Monika Hilker [b,*], Axel Schmidt [a]

[a] Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll-Str. 8, D-07745 Jena, Germany
[b] Institute of Biology, Freie Universität Berlin, Haderslebener Str. 9, D-12163 Berlin, Germany

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ABSTRACT

Pinus sylvestris (Scots pine) is known to respond to eggs laid by the sawfly *Diprion pini* on its needles by releasing a blend of terpenoids, including the sesquiterpene (*E*)-β-farnesene. These compounds attract a wasp, *Closterocerus ruforum*, which parasitizes sawfly eggs. *D. pini* oviposition also enhances the transcription of two sesquiterpene synthases, an (*E*)-β-caryophyllene/α-humulene synthase (*PsTPS*1) and a 1(10),5-germacradiene-4-ol synthase (*PsTPS*2). To gain a better understanding of the function of these sesquiterpenes in promoting insect egg parasitism, we compared the outcome of *D. pini* oviposition on *P. sylvestris* with interactions between other pine and sawfly species: *Neodiprion sertifer* eggs on *P. sylvestris*, *Gilpinia pallida* eggs on *P. sylvestris*, *D. pini* eggs on *Pinus nigra*. The first of these attracts the parasitoid *C. ruforum*, while the latter two do not. As determined by quantitative real-time PCR, both *PsTPS*1 and *PsTPS*2 transcripts increased significantly only for those species combinations where the odor of egg–laden pine needles was attractive to *C. ruforum*. Moreover, enhanced transcription of these genes was found only at those time periods when odor was attractive, i.e. 3 days after oviposition. Thus, the *PsTPS*1 and *PsTPS*2 genes are good markers for parasitoid attraction. We also characterized a sesquiterpene synthase from *P. sylvestris* (*PsTPS*5) which produces (*E*)-β-farnesene, the compound previously determined to be responsible for *C. ruforum* attraction. However, transcript levels of *PsTPS*5 were not enhanced by oviposition of sawfly species that cause *C. ruforum* attraction. More research on this experimental system is required to determine the role of oviposition-induced sesquiterpenes in attracting egg parasitoids and the role of sesquiterpene synthases in regulating sesquiterpene formation.

1. Introduction

Plants are well known to activate a wide range of defense mechanisms in response to attack by herbivorous arthropods (Karban and Baldwin, 1997; Walling, 2000). One common mechanism is the emission of volatiles that attract parasitic wasps. Some parasitic wasp species infest larval stages of herbivorous insects, and their attraction to plant volatiles released during feeding has been frequently studied (D'Alessandro and Turlings, 2006; Hilker and Meiners, 2006; Dicke, 2009). Other parasitic wasps infest herbivore eggs, and their attraction to plant volatiles released on egg laying has been shown for plant species, such as the elm *Ulmus minor* (Meiners and Hilker, 2000), the pine *Pinus sylvestris* (Hilker et al., 2002a,b; Mumm and Hilker, 2006; Hilker and Meiners, 2009), and the bean plants *Vicia faba* and *Phaseolus vulgaris* (Colazza et al., 2004a,b). In the case of *P. sylvestris*, terpene blends are emitted both before and after oviposition by the sawfly, *Diprion pini*. However, the sesquiterpene, (*E*)-β-farnesene, was shown to be emitted at higher levels after oviposition (Mumm et al., 2003) and was demonstrated to be attractive to the egg parasitoid, *Closterocerus* (formerly *Chrysonotomyia*) *ruforum* when offered against the background of volatiles from *P. sylvestris* (Mumm and Hilker, 2005). Among the necessary background odor components for this attraction are the sesquiterpenes, (*E*)-β-caryophyllene and α-humulene (Beyaert et al., 2010).

In order to learn more about what regulates the production of these egg parasitoid-attracting sesquiterpenes in *P. sylvestris*, we initiated an investigation of the sesquiterpene synthases of this species (Köpke et al., 2008). These enzymes convert (*E*,*E*)-farnesyl diphosphate, the ubiquitous, linear C_{15} intermediate of terpene metabolism, into a wide range of sesquiterpene carbon skeletons. Three *P. sylvestris* sesquiterpene synthases were characterized in earlier work (Köpke et al., 2008). *Ps*TPS1 catalyzes the formation of (*E*)-β-caryophyllene and α-humulene. *Ps*TPS2 catalyzes the production of 1(10),5-germacradiene-4-ol as a major product, with minor amounts of bicyclogermacrene, α-amorphene and germacrene A. *Ps*TPS3 forms longifolene as a major product with minor amounts of α-longipinene, α-ylangene, longiborneol and longicyclene. However, no enzyme has been discovered which forms (*E*)-β-

Abbreviations: FPP, farnesyl disphosphate; GPP, geranyl diphosphate; GGPP, geranyl geranyl diphosphate.

* Corresponding author. Tel.: +49 30 838 55913; fax: +49 30 838 53897.
E-mail address: hilker@zedat.fu-berlin.de (M. Hilker).

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farnesene, the major attractant of *C. ruforum*. In studying the expression of the isolated genes, *PsTPS*1 and *PsTPS*2 were found to have significantly higher transcript levels in *P. sylvestris* needles with *D. pini* eggs that were attractive to *C. ruforum* than in non-attractive, artificially-damaged needles.

To study the importance of chemical signals in biological interactions, it is often valuable to make comparisons among closely related species. For example, in addition to *D. pini*, the sawfly *Neodiprion sertifer* also lays its eggs and feeds on *P. sylvestris* foliage, and the volatiles attract the eulophid wasp *C. ruforum* (Mumm and Hilker, 2005). Another sawfly, *Gilpinia pallida*, lays its eggs on *P. sylvestris* as well and is a host for *C. ruforum*, but the volatiles released are not attractive to this egg parasitoid. When the host plant *P. sylvestris* is switched for *Pinus nigra*, *D. pini* will still lay eggs and feed on the needles of this pine species. However, the odor of *P. nigra* laden with *D. pini* eggs is unattractive to *C. ruforum*, possibly because the performance of this egg parasitoid is reduced compared to its performance on *D. pini* eggs laid on *P. sylvestris* (Auger et al., 1994; Barre et al., 2002). Thus, the attractiveness of pine odor induced by sawfly oviposition for the egg parasitoid *C. ruforum* is specific to certain combinations of pine and sawfly species.

In this study, we took advantage of the species specificity of interactions between pine, sawflies, and parasitoids to assess the importance of sesquiterpene synthases for the attraction of *C. ruforum*. Following our work on *P. sylvestris* with *D. pini* egg depositions, we report here the transcript levels of the pine sesquiterpene synthases, *PsTPS*1 and *PsTPS*2 in three other pairwise interactions between pine and sawfly species, some of which produce an odor attractive to the egg parasitoid *C. ruforum* and some which do not. We also describe a new terpene synthase that produces (*E*)-β-farnesene and examine its transcript levels during pine–sawfly interactions.

Before carrying out these experiments, it was first necessary to conduct behavioral assays with *C. ruforum* to determine the timing of its attraction to twigs upon which sawfly eggs had been laid. Previous results had shown that, when *D. pini* oviposited on *P. sylvestris*, the volatile blend was attractive 3 days after oviposition, but not before or after that time (Köpke et al., 2008). However, for the other combinations of pine and sawfly species, we first had to determine the timing of peak volatile attraction before conducting sesquiterpene transcript analyses since this had only been studied at 3 days after oviposition in each case (Table 1).

Table 1
Combinations of pine and sawfly species tested in previous studies for attraction of egg parasitoids to pine odor induced by sawfly egg deposition.

Pine species	Sawfly species	Time after egg deposition (day)	Response by egg parasitoids[a]	Reference
P. sylvestris	*D. pini*	2	No attraction	Köpke et al. (2008)
		3	Attraction	Hilker et al. (2002a), Köpke et al. (2008)
		4	No attraction	Köpke et al. (2008)
P. sylvestris	*N. sertifer*	3	Attraction	Mumm et al. (2005)
P. sylvestris	*G. pallida*	3	No attraction	Mumm et al. (2005)
P. nigra	*D. pini*	3	No attraction	Mumm et al. (2005)

[a] Egg parasitoid tested: *Closterocerus* (formerly *Chrysonotomyia*) *ruforum*.

2. Results and discussion

2.1. The attraction of C. ruforum to pine odor at different times after sawfly egg deposition

The olfactory response of female *C. ruforum* egg parasitoids to pine laden with eggs of various sawfly species was tested 2, 3 or 4 days after oviposition using a four-field olfactometer. We assessed whether parasitoids spent significantly longer walking in a test field supplied with odor compared with control fields. Previous studies had shown attraction or non-attraction 3 days after egg deposition, but tests had been conducted on other days for only *D. pini* on *P. sylvestris* twigs (Table 1). These results showed that the *P. sylvestris* twigs with *D. pini* eggs were attractive 3 days after oviposition, but not 2 or 4 days afterwards. Odor from *P. sylvestris* laden with *D. pini* eggs for 2 days elicited tentatively a positive response by the egg parasitoids. However, in spite of a high number of replicates, no significant attraction was recorded.

In the present work, when *P. sylvestris* twigs were tested with *N. sertifer* eggs, the pattern of response was the same with attraction evident only at 3 days, but not at 2 or 4 days after oviposition (Table 2). However, *P. sylvestris* twigs laden with *G. pallida* eggs did not show a significant attraction to *C. ruforum* at any time point tested. Finally, *P. nigra* twigs with *D. pini* eggs were also not attractive at any time tested. Whether the composition of volatile blends emitted by pine twigs changes over this time period is not known for most of these combinations besides *P. sylvestris–D. pini* (Mumm et al., 2003; Mumm and Hilker, 2005). The temporal patterns of parasitoid attraction may be a result of when the sawfly egg is most suitable for infestation. For example, the eggs of the stink bug, *Nezara viridula*, laid on bean plants were suggested to be too old for successful infestation of the egg parasitoid, *Trissolcus basalis*, 96 h after oviposition, when hatching was imminent (Colazza et al., 2004a).

2.2. The effect of oviposition on transcript levels of the sesquiterpene synthases TPS1 and TPS2

To investigate the role of pine sesquiterpene synthases in the attraction of egg parasitoids to sawfly eggs, we studied the transcript levels of two sesquiterpene synthase genes that were induced by oviposition in our previous studies (Köpke et al., 2008) in three pairwise species interactions between pine and sawflies, one attractive to *C. ruforum* and two unattractive. *P. sylvestris* needles on which *N. sertifer* eggs had been laid 3 days previously were attractive to the egg parasitoid, *C. ruforum* (Table 2). The transcript levels of *PsTPS*1 and *PsTPS*2 in these needles were significantly higher than levels of these genes in egg-free control needles not attractive to the parasitoids (Fig. 1). On average, transcript levels were 17.5-fold higher for *PsTPS*1 in egg–laden needles and 30.5-fold higher for *PsTPS*2. At 2 days after oviposition, transcript levels of these genes were not significantly different from those in control needles, and at 4 days after oviposition transcripts were significantly reduced compared to the controls. Thus, *PsTPS*1 and *PsTPS*2 in *P. sylvestris* with *N. sertifer* eggs showed enhanced transcription only at the time when pine odor was attractive to the egg parasitoids (Tables 1 and 2). These results are very similar to those we obtained previously on the transcript levels of these genes in *P. sylvestris* laden with the eggs of *D. pini* (Köpke et al., 2008).

Other combinations of pine and sawfly species gave different results. Analysis of *P. sylvestris* needles on which the sawfly *G. pallida* had oviposited did not show any significant increase in transcripts of *PsTPS*1 and *PsTPS*2 at any time point tested (Fig. 1), consistent with the unattractiveness of odor released by this combination of pine and sawfly species (Tables 1 and 2). When *P. nigra*