Indirect defence of *Arabidopsis* against herbivorous insects

Combining parasitoid behaviour and chemical analyses with a molecular genetic approach

Promotor:

Prof. dr. M. Dicke
Hoogleraar in de insect-plant relaties,
in het bijzonder tritrofe interacties

Promotiecommissie

Prof. dr. ir. M. Koornneef	Wageningen Universiteit
Prof. dr. L.H.W. van der Plas	Wageningen Universiteit
Prof. dr. L.E.M. Vet	Wageningen Universiteit
Prof. dr. L.C. van Loon	Universiteit Utrecht

Remco M. P. van Poecke

Indirect defence of *Arabidopsis* against herbivorous insects

Combining parasitoid behaviour and chemical analyses with a molecular genetic approach

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op vrijdag 22 november 2002
des namiddags te vier uur in de Aula

Van Poecke, Remco M. P.

Indirect defence of *Arabidopsis* against herbivorous insects: Combining parasitoid behaviour and chemical analyses with a molecular genetic approach

Thesis Wageningen University - with references - with summary in Dutch

ISBN: 90-5808-698-4

Contents

Abstract

Many plant species are known to defend themselves against herbivorous insects indirectly, by producing volatiles in response to herbivory. These volatiles attract carnivorous enemies of the herbivores, such as parasitoids. Research on the model plant *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) has contributed considerably to the unravelling of signal transduction pathways involved in *direct* plant defence mechanisms against pathogens. In this thesis I demonstrate that *Arabidopsis* is also a good model plant for studying signal transduction pathways involved in *indirect* defence mechanisms, by showing that: (a) *Arabidopsis* plants infested by *Pieris rapae* caterpillars (Lepidoptera: Pieridae) emit volatiles from several major biosynthetic pathways, including terpenoids, methyl-salicylate (MeSA), nitriles and green leaf volatiles; (b) Adult females of *Cotesia rubecula* (Hymenoptera: Braconidae), a specialist parasitoid wasp of *P. rapae*, were attracted to *P. rapae*-infested *Arabidopsis* plants; and (c) Genes from major biosynthetic pathways involved in volatile production were induced by caterpillar feeding.

Moreover, I show that the octadecanoid and the salicylic acid pathways are involved in the induced attraction of *C. rubecula* by *P. rapae*-infested *Arabidopsis*. Besides exogenous application of jasmonic acid (JA) or salicylic acid (SA), I used transgenic *Arabidopsis* that do not show induced JA levels after wounding (S-12) and transgenic *Arabidopsis* that do not accumulate SA (NahG). Treatment of *Arabidopsis* with JA resulted in an increased attraction of parasitoid wasps compared to untreated plants, whereas treatment with SA did not. Transgenic plants impaired in the octadecanoid or the salicylic acid pathway were less attractive than wild-type plants. Chemical analysis of the volatile blends emitted by JA-treated wild-type and *P. rapae*-infested wild-type, S-12 and *NahG* plants, demonstrate that JA and SA are indeed involved in the herbivory-induced production of volatiles. Moreover, these data suggest important roles for MeSA and the terpenoid (3*E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene as parasitoid attractants. Additionally, these data suggest a signalling role of the plant hormone 12-oxo-phytodienoic acid in induced volatile emissions.

Although *direct* defence mechanisms against pathogens and herbivores are often also regulated through JA and SA, these signal transduction pathways differ from those involved in *indirect* defence of *Arabidopsis* against *P. rapae*, as I demonstrated by using the signal transduction mutants *npr1-1* and *jar1-1*.

In this thesis it is also shown that herbivore species with a different way of feeding compared to *P. rapae* caterpillars - aphids and spider mites - induced no or less attraction of *C. rubecula* when infesting *Arabidopsis*. This difference in parasitoid attraction may be the result of different induction of JA and SA signalling pathways by different herbivore species.

In conclusion, this thesis demonstrates that combing parasitoid behaviour and chemical analysis with a molecular genetic approach can be highly valuable in unravelling signal-transduction pathways involved in indirect defence of plants, a method that so far has been under-exploited.

Preface

On Monday morning, the 5th of January 1998, I entered the world of insects. Although they sometimes bugged me big time, I never regretted my decision to work with these tiny creatures. The four and a half years I have spent working on this thesis have (mostly) been a pleasure indeed. A great contributor to this fact is my promotor Marcel, who introduced me to the field of plant-insect interactions. His enthusiasm and confidence were very motivating, his speed in reviewing my manuscripts incredible. Although in the last months of this project I tried to write quicker than he could review, I did not succeed.

When I started working in the Lab of Entomology, together with Rieta I had the honour of being the first inhabitant of the Goedaert room and many a story was told in that room. Nowadays, we leave most of the talking to Jeff and concentrate on sinking balls. In times when I was not too pleased with the way my pets - both wasps and plants - were behaving or cooperating, I could count on my colleagues for moral support. The first to see my chagrined head were often my roommates - Marieta, Clemens, Karin and Sander. If they could not console me, the neighbours - especially Sara, Joep, Antoon and Jet - could, with loads and loads of coffee.

Working on a PhD project is nice, but even better when there are students to help you. Mara and Lorena, I enjoyed our working together very much. Not only students helped me with acquiring and/or analysing data. Maarten performed the chemical analysis of the volatiles I collected, without him a major pillar of this thesis would not have been there.

What would a lab of Entomology be without insects? The rearing of many different species was in the capable hands of André, Frans, Leo and Herman, thank you! I want to express the same gratitude to Casper and Aart. Thanks to them, a steady flow of *Arabidopsis* plants came my way.

Colleagues were also part of life outside the lab. Whether it was hiking on the Veluwe together with Joep and Cindy at ridiculous times, ice-skating with Ellis, Marnix, Olga, Cindy, Joke, Jet and E, or drinking beer in the 'surprise pub' with William, Cindy and Jet, we had lots of fun. Of course there are many more people that made life at Ento so good. However, this preface is already becoming way to long, so for all the colleagues not mentioned above: thank you!

Although I enjoy working in science very much, the happiest and saddest moments took place outside the 'scientific life'. Hanneke, you were with me on many of these moments. Our six years together were wonderful. You will always be in my heart and mind.

Many other friends surround me who make life worthwhile: Tom, Joop, E, Luc, Vera, Lidwine, Tonny, Marc, Margo, Margot, Marrit, Morris, Ab, and many more.

And then there's the home front. Pa and Ma, you have made it all possible in many different ways. Thank you for letting me live the life I choose, for your support, and your confidence in me. Sas, Jos, and the rest of the family, you are part of me in a way only family can be.

Darn, I didn't succeed in keeping this preface on one page. There is, however, still someone left who, although already mentioned a couple of times, deserves a few more lines. Lover, partner, colleague, friend: Jet, I cannot thank you enough. Your love, patience, support and advice have helped me through the last one-and-a-half year. I could not have done it without you!

This leaves me nothing but to hope that you, the readers, will enjoy reading this thesis as much as I have enjoyed working on it.

Remco van Poecke
Wageningen, August 2002

Voor Pa en Ma

chapter
1

General introduction

Remco M.P. van Poecke

"Plants, whether very simple or highly complex, have colonised almost the whole of the surface of our planet, from the snows of the Poles to the lush rain forests on the equator. Some tolerate far harsher circumstances than any animal can. They live in oven-hot conditions where animals can only briefly venture, and survive in extremes of cold that would freeze an animal to death.

Only one thing they cannot survive - the determined onslaughts of humanity. Ever since we appeared on earth as a species, we have dug them up and cut them down, burnt them and poisoned them. Today, we are doing so on a greater scale than ever before. We destroy them at our peril. Without them, neither we, nor any other animal can exist. It is time now for us to cherish our green inheritance, not to pillage it.

Without it we will surely perish."

David Attenborough - The private life of plants

Chapter 1

GENERAL INTRODUCTION

1. The struggle for life

Plants live a hard life. As soon as they sprout from seed, they have to face heat, cold, drought, flooding, wind, the burning of the sun or a lashing by rain. And if they are not trampled by cattle or plagued by diseases, insects may nibble at their leaves, stems, roots, or flowers. Whilst facing these attacks, they also have to acquire water, nutrients, and often pollinators to produce new seeds. Plants cannot run away from attacks or move to more suitable locations. To survive, a plant must adapt and defend itself (Nagy and Schäfer, 2002; Scheel and Wasternack, 2002).

Some of the defence mechanisms of plants are permanently present, even if there is nothing there to defend against. These so-called constitutive defences form the first wall against attack or unfavourable environmental conditions. Such defences require water, nutrients, light and CO_2 from their environment. This requirement also illustrates a disadvantage of these constitutive defences: they are costly. The nutrients and energy invested in constitutive defences can usually not be used by the plant for growth or reproduction (Karban and Baldwin, 1997; Baldwin, 1998).

2. Inducible defences

The costs of constitutive defence are likely one of the reasons why, besides constitutive defences, most if not all plants also employ inducible defences. These defences accumulate when the plant needs them (Baldwin, 1998). Examples are the accumulation of sugars as a defence against freezing (Salinas, 2002) and the production of toxins in response to herbivory (Karban and Baldwin, 1997). Although the reduction of costs is a likely stimulus for the evolution of inducible defences there are additional explanations. For example, herbivores that are continuously exposed to toxins are more likely to adapt to these toxins than herbivores that encounter variable levels of these toxins (Agrawal and Karban, 1999).

If inducibility of defences has so many advantages, why then are not all defences inducible? Apparently there are also disadvantages to inducible defences. One of these is that it takes time to build up inducible defences. If there are no constitutive defences present, the plants might suffer substantial damage before the inducible defences can accumulate (Baldwin, 1998).

3. Signal transduction

In contrast to constitutive defences, inducible defences require additional interactions with the environment in addition to resources to develop the defences. The plant needs to sense changes in the environment, whether this is a temperature drop, attack by herbivores, or other changes (Karban and Baldwin, 1997; Reymond *et al.*, 2000; Salinas, 2002; Schaller and Weiler, 2002). Upon perception of these changes, this information has to be processed, ultimately leading to responses that give (some) protection. The processing of these signals is called signal transduction, and the pathways in the plant through which this occurs are called signal-transduction pathways (Scheel and Wasternack, 2002).

Many signal-transduction pathways depend on the action of plant hormones. Several plant hormones have been identified and their role in defence signalling has been established. Examples of these plant hormones are jasmonic acid and salicylic acid (Creelman and Mullet, 1997; Métraux, 2001). However, there are many more different defence responses than there are plant hormones, meaning that not each different defence response can require a different hormone. Besides this, many plant hormones do not only function in defence responses, but also play a role in other processes, such as plant growth and flower development (Creelman and Mullet, 1997). One way of achieving such a diversity of responses with a limited number of plant hormones is through interactions between the different hormones. If a certain response requires the combined action of several hormones, many more different responses are possible with a limited number of plant hormones. This also implies that different responses can share the same plant hormone and as such, different responses can influence each other, either positively or negatively. Although many signalling compounds (such as plant hormones) are known, and their interaction with other components of the signal-transduction pathway is sometimes partially understood, the way in which the different signal-transduction pathways interact and how this leads to the diversity of responses remains largely unknown.

4. Indirect defence

The defence of plants against insect herbivores involves many different responses. Some of these have a direct effect on the herbivore, like the production of toxins or repellents (Schoonhoven *et al.*, 1998). Others affect the herbivores in an indirect way, by making use of the carnivorous enemies of the herbivores. These carnivores can be predators or parasitoids. Predators eat the herbivores or the eggs of the herbivores; parasitoids lay their eggs in or on the herbivores or the eggs of the herbivores. When a parasitoid egg hatches, the larva starts feeding on the herbivore or on the egg of the herbivore. The plant can enhance the effectiveness of these carnivores, for example by supplying them with food or shelter, or by providing them with information on the presence of herbivores (Janzen, 1966; Dicke, 1999; Wackers and Wunderlin, 1999). Herbivores are under selection to avoid being easily detected by carnivores. Plants can help the carnivores locating their herbivorous victim. They can do this by producing volatile compounds in response to herbivore feeding. This information can be so detailed that it also tells which herbivore species is feeding on the plant. This is important for the carnivores, as many of the carnivores are specialists that feed on only one or a few herbivore species (Vet and Dicke, 1992; Dicke and Vet, 1999; Pare and Tumlinson, 1999).

As the production of these carnivore-attracting volatiles is an induced response (induced by the feeding of the herbivore), it requires perception of feeding damage and the processing of the signal through signal-transduction pathways. Knowledge on factors from the herbivore involved in the elicitation of volatiles is increasing. First of all, herbivory by insects often involves wounding of plant tissue. This wounding can elicit volatile production. Secondly, compounds in the oral secretions (regurgitant) of several caterpillar species can also elicit volatile production (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Halitschke *et al.*, 2001).

Knowledge on how plants perceive these signals and how they are transduced is limited. The plant hormone jasmonic acid, known for its importance in induced plant responses to wounding and induced direct defence against insects (Creelman and Mullet, 1997; McConn *et al.*, 1997; Thaler *et al.*, 2001; Karban and Baldwin 1997), is also involved in induced emission of volatiles (Piel *et al.*, 1997; Dicke *et al.*, 1999; Koch *et al.*, 1999). Recently, a role for salicylic acid, involved in the induced defence against many pathogens (Dong, 2001; Métraux, 2001), in herbivory-induced volatile production has been suggested (Ozawa *et al.*, 2000). How these plant hormones affect volatile production is not known yet.

5. *Arabidopsis thaliana* as a model plant

To get a better understanding of these mechanisms, this thesis introduces the plant *Arabidopsis thaliana* into this research field. *Arabidopsis* is a member of the Brassicaceae (=crucifer) plant family and as such related to crop plants such as cabbage and mustard. It is often used in studies on signal-transduction pathways, including those involved in induced plant defence. This has several reasons. First of all, it is easy to rear. The entire life cycle from seed germination to flowering and the production and ripening of new seeds can be completed in six weeks. The plant is small with a simple structure, does not have complicated requirements for growth, produces thousands of seeds per plant and is easy to cross. Secondly, it has a small diploid genome of which the entire sequence is known. This genome is easily manipulated through mutation or transformation. Thirdly, *Arabidopsis* is widely distributed in Eurasia, North Africa and North America resulting in many genetically different ecotypes. Fourthly, *Arabidopsis* is attacked by a wide range of herbivorous insects and microbial pathogens (Meyerowitz and Somerville, 1994; Meinke *et al.*, 1998; Mitchell-Olds, 2001).

These many advantages have resulted in an enormous variety of wild-type, mutant and transgenic plants, including some that are altered or inhibited in certain signal-transduction pathways. These mutant and transgenic plants are highly valuable for studies on signal-transduction pathways (Glazebrook, 2001). Indeed, signal transduction in induced defences of *Arabidopsis* against pathogens has been well studied (Dong, 2001; Pieterse *et al.*, 2001; Glazebrook, 2001). Some first studies on induced defence against herbivorous insects have emerged in recent years (McConn *et al.*, 1997; Mauricio, 1998; Stotz *et al.*, 1999; Van Loon *et al.*, 2000; Reymond *et al.*, 2000).

In addition to these many advantages, the large amount of information on signal transduction in induced responses in *Arabidopsis* enables us to compare the results that we obtained by studying signal-transduction pathways involved in indirect defence with studies on other signal-transduction pathways, for example those induced by wounding or pathogen infection. For a useful comparison with these different signal-transduction pathways it is important to use the same plant species, as different plant species might differ in signal transduction.

6. Plant-herbivore-parasitoid interactions: *Arabidopsis-Pieris-Cotesia*

Crucifers are attacked by several herbivorous insects and interactions between crucifers and *Pieris* spp. (Lepidoptera) have been well studied (Blaakmeer *et al.*, 1994; Agrawal, 1999; Renwick and Lopez, 1999). *Pieris* caterpillars are well known to induce indirect defences in crucifers: for example caterpillar damage to *Brassica oleracea* results in the emission of herbivore-induced volatiles that attract *Cotesia* parasitoids (Geervliet *et al.*, 1994; Mattiacci *et al.*, 1994). In this study I have mainly worked with caterpillars of the small cabbage white (*Pieris rapae*) and the parasitoid *Cotesia rubecula*.

Pieris rapae caterpillars only feed on plants that contain glucosinolates, which are mainly cruciferous plants including *Arabidopsis*. *Pieris rapae* is common in Eurasia, North Africa, North America and Australia, so there is a large overlap in geographical distribution between *P. rapae* and *Arabidopsis*. *Pieris rapae* caterpillars have been found on *Arabidopsis*. *Pieris rapae* is a solitary species, which means that the butterflies lay eggs singly (instead of in a clutch), often resulting in only one egg per plant (Geervliet, 1997).

The parasitoid *Cotesia rubecula* is a specialist parasitoid of *P. rapae* caterpillars (Harvey *et al.*, 1999). The parasitoid mainly uses plant volatiles to find its host from a distance (Geervliet *et al.,* 1994). Out of the five caterpillar instars, it can successfully parasitize the first, second, and to a lesser extent third stage. It is a solitary parasitoid, which means that only one parasitoid can successfully develop per caterpillar. The larvae of *C. rubecula* egress from the caterpillars when these are in the fourth larval stage, after which the parasitoid larvae pupate outside of the host. The caterpillars die soon after the parasitoid larvae have egressed. This is beneficial for the plant, as unparasitized *P. rapae* caterpillars eat most when they are in the fifth larval stage (Harvey *et al.*, 1999; Harvey, 2000). Therefore, the plant receives less damage and can produce more seeds when it is fed upon by a parasitized *P. rapae* caterpillar compared to a caterpillar that was not parasitized by *C. rubecula*. *Arabidopsis* was the first plant species for which such a benefit in seed production has been demonstrated (Van Loon *et al.*, 2000).

7. Questions addressed in this thesis

Summarizing, we know that plants can defend themselves against herbivorous insects indirectly by attracting carnivores such as parasitoids. As this is an inducible defence mechanism it requires signal transduction in the plant. Knowledge about this signal transduction in induced plant volatiles is limited, but jasmonic acid and salicylic acid are likely to be involved. These plant hormones are also involved in induced direct defence against insects and pathogens. The use of *Arabidopsis thaliana*, an important model plant in contemporary plant sciences, is ideal for a molecular genetic approach to study signal transduction involved in indirect defences against herbivores and to study how this signal transduction is related to signal transduction in direct defences. The value of a molecular genetic approach in studies on insect-plant interactions has been pointed in several papers (Mitchell-Olds *et al.*, 1998; Kessler and Baldwin, 2002, Dicke *et al.*, 2002). This thesis reports the first results obtained by using this method for studying indirect plant defences. In this thesis I address the following questions:

1. Is *Arabidopsis* a suitable model plant to study indirect defence?
2. Are the plant hormones jasmonic acid (JA) and salicylic acid (SA) involved in the signal transduction of indirect defences of *Arabidopsis* and if so, how are these hormones involved?
3. What happens downstream of jasmonic acid and salicylic acid in the signal transduction of indirect defences and how does this relate to other plant defences of *Arabidopsis*, such as those against pathogens?

8. Thesis outline

Before addressing these questions, **chapter 2** gives a review of the literature on signal transduction in induced direct and indirect defences of plants against herbivores.

Question 1: Is Arabidopsis a suitable model plant to study indirect defence?

In **chapter 3** we address this question using undamaged, artificially damaged and *P. rapae*-infested wild-type *Arabidopsis* plants. We studied the attraction of *C. rubecula* to these differently treated plants in a two choice set up. Moreover, we analysed the chemical composition of the volatile blends emitted by these plants. The results tell us whether *Arabidopsis* does emit herbivory-induced volatiles that can attract parasitoids. Additionally we studied the expression of several genes involved in volatile production and signal transduction. These genes include genes involved in JA synthesis, genes involved in SA synthesis, and genes involved in the production of several groups of volatiles, including green leaf volatiles and terpenoids.

Chapters 4 and 5 address whether JA-treatment of *Arabidopsis* has a similar effect on volatile emission and carnivore attraction compared to other plant species, using behavioural and chemical analysis.

Whether herbivore-derived elicitors are involved in the induction of volatiles that attract *C. rubecula* to herbivore-infested *Arabidopsis* is discussed in **chapter 6**.

Whether *C. rubecula* responds to *Arabidopsis* plants infested by different herbivore species in a similar fashion compared to other brassicaceous plant species infested by different herbivore species is discussed in **chapter 7**.

Question 2: Are the plant hormones jasmonic acid (JA) and salicylic acid (SA) involved in the signal transduction of indirect defences of Arabidopsis?

This question is addressed in **chapters 4 and 5**. Besides using exogenous application of JA and SA on wild-type plants, we also studied the volatile emission of and parasitoid attraction to transgenic *Arabidopsis* plants that either are unable to accumulate SA (Delaney *et al.*, 1994) or do not show wound-induced accumulation of JA (Bell *et al.*, 1995). The expression of several genes that were induced by *P. rapae*-feeding in wild-type plants (**chapter 3**), was studied in these transgenic plants.

How JA and SA are involved in the differential attraction of *C. rubecula* by *Arabidopsis* plants infested by different herbivore species is discussed in **chapter 7**.

Question 3: What happens downstream of jasmonic acid and salicylic acid in the signal transduction of indirect defences and how does this relate to other plant defences of Arabidopsis, such as those against pathogens?

This question is addressed in **chapter 6** where we studied the behaviour of *C. rubecula* to *P. rapae*-infested mutant *Arabidopsis* plants that are affected in signal transduction downstream of JA and SA.

9. References

Agrawal, A.A. (1999). Induced responses to herbivory in wild radish: Effects on several herbivores and plant fitness. *Ecology* 80: 1713-1723.

Agrawal, A.A. and Karban, R. (1999). Why induced defenses may be favored over constitutive strategies in plants. In *The Ecology And Evolution Of Inducible Defenses*, Tollrian R. and Harvell, C.D. (eds). Princeton University Press, Princeton, USA, pp. 45-61.

Alborn, T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H. and Tumlinson, J.H. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. Science 276: 945-949.

Baldwin, I.T. (1998). Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. USA* 95: 8113-8118.

Bell, E., Creelman, R.A. and Mullet, J.E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*. 92: 8675-8679.

Blaakmeer, A., Hagenbeek, D., Van Beek, T.A., De Groot, A.E., Schoonhoven, L.M., Van Loon, J.J.A. (1994). Plant response to eggs vs. host marking pheromone as factors inhibiting oviposition by *Pieris brassicae*. *J. Chem. Ecol.* 20: 1657-1665.

Creelman, R.A. and Mullet, J.E. (1997). Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant. Mol. Biol.* 48: 355-381.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut Rella, M., Kessmann, H., Ward, E. Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. *Science* 266: 1247-1250.

Dicke, M. (1999). Evolution of induced indirect defense of plants. In *The Ecology And Evolution Of Inducible Defenses*, Tollrian R. and Harvell, C.D. (eds). Princeton University Press, Princeton, USA, pp. 62-88.

Dicke, M., Gols, R., Ludeking, D. and Posthumus, M.A. (1999). Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25: 1907-1922.

Dicke, M., Van Poecke, R.M.P. and de Boer, J.G. (2002) Inducible indirect defence of plants: from subcellular mechanisms to ecological functions. *Basic Appl. Ecol.*, in press.

Dicke, M. and Vet, L.E.M. (1999). Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore. In *Herbivores Between Plants And Predators*, Olff, H. Brown, V.K. and Drent, R.H. (eds). Blackwell Science, Oxford, UK, pp. 483-520.

Dong, X.N. (2001). Genetic dissection of systemic acquired resistance. *Curr. Opin. Plant Biol.* 4: 309-314.

Geervliet, J.B.F. (1997). *Infochemical use by insect parasitoids in a tritrophic context: comparison of a generalist and a specialsist. PhD Dissertation.* Wageningen Agricultural University, Wageningen, The Netherlands.

Geervliet, J.B.F., Vet, L.E.M. and Dicke, M. (1994). Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomol. Exp. Appl.* 73: 289-297.

Glazebrook, J. (2001). Genes controlling expression of defense responses in *Arabidopsis* - 2001 status. *Curr. Opin. Plant Biol.* 4: 301-308.

Halitschke, R., Schittko, U., Pohnert, G., Boland, W. and Baldwin, I.T. (2001). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiol.* 125: 711-717.

Harvey, J.A. (2000). Dynamic effects of parasitism by an endoparasitoid wasp on the development of two host species: implications for host quality and parasitoid fitness. *Ecol. Entomol.* 25: 267-278.

Harvey, J.A., Jervis, M.A., Gols, R., Jiang, N. and Vet, L.E.M. (1999). Development of the parasitoid, *Cotesia rubecula* (Hymenoptera: Braconidae) in *Pieris rapae* and *Pieris brassicae* (Lepidoptera: Pieridae): Evidence for host regulation. *J. Insect Physiol.* 45: 172-182.

Janzen, D.H. (1966). Coevolution of mutualism between ants and acacias in Central America. *Evolution* 20: 249-275.

Karban, R. and Baldwin, I.T. (1997). *Induced Responses to Herbivory.* The University of Chicago Press Ltd, Chicago, USA.

Kessler, A. and Baldwin, I.T. (2002). Plant responses to insect herbivory: the merging molecular analysis. *Annu. Rev. Plant Biol.* 53: 299-328.

Koch, T., Krumm, T., Jung, V., Engelberth, J. and Boland, W. (1999). Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid signaling pathway. *Plant Physiol.* 121: 153-162.

Mattiacci, L., Dicke, M. and Posthumus, M.A. (1994). Induction of parasitoid attracting synomone in Brussels sprouts plants by feeding of *Pieris brassicae* larvae: role of mechanical damage and herbivore elicitor. *J. Chem. Ecol.* 20: 2229-2247.

Mattiacci, L., Dicke, M. and Posthumus, M.A. (1995). b-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proc. Natl. Acad. Sci. USA* 92: 2036-2040.

Mauricio, R. (1998). Costs of resistance to natural enemies in field populations of the annual plant *Arabidopsis thaliana*. *American Naturalist* 151: 20-28.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E. and Browse, J. (1997). Jasmonate is essential for insect defense *Arabidopsis. Proc. Natl. Acad. Sci. USA* 94: 5473-5477.

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D. and Koornneef, M. (1998). *Arabidopsis thaliana*: A model plant for genome analysis. *Science* 282: 662-682.

Métraux, J.P. (2001). Systemic acquired resistance and salicylic acid: current state of knowledge. *Eur. J. Plant Pathol.* 107: 13-18.

Meyerowitz, E.M. and Somerville, C.R. (1994). *Arabidopsis.* Cold Spring Harbor Laboratory Press, New York, USA.

Mitchell Olds, T. (2001). *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. *Trends Ecol. Evol.* 16: 693-700.

MitchellOlds, T., Gershenzon, J., Baldwin, I. and Boland, W. (1998). research focus - Chemical ecology in the molecular era. *Trends Plant Sci.* 3: 362-365.

Nagy, F. and Schäfer, E. (2002). Light perception and signal transduction. In *Plant Signal Transduction*, Scheel D. and Wasternack C. (eds). Oxford University Press, Oxford, UK, pp. 6-19.

Ozawa, R., Arimura, G., Takabayashi, J., Shimoda, T. and Nishioka, T. (2000). Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. *Plant Cell Physiol.* 41: 391-398.

Pare, P.W. and Tumlinson, J.H. (1999). Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121: 325-331.

Piel, J., Atzorn, R., Gabler, R., Kuhnemann, F. and Boland, W. (1997). Cellulysin from the plant parasitic fungus *Trichoderma viride* elicits volatile biosynthesis in higher plants via the octadecanoid signalling cascade. *FEBS-Letters* 416: 143-148.

Pieterse, C.M.J., Van Pelt, J.A., Van Wees, S.C.M., Ton, J., Leon Kloosterziel, K.M., Keurentjes, J.J.B., Verhagen, B.W.M., Knoester, M., Van der Sluis, I., Bakker, P.A.H.M, and Van Loon, L.C. (2001). Rhizobacteria-mediated induced systemic resistance: Triggering, signalling and expression. *Eur. J. Plant Pathol.* 107: 51-61.

Renwick, J.A.A. and Lopez, K. (1999). Experience-based food consumption by larvae of Pieris rapae: addiction to glucosinolates? *Entomol. Exp. Appl.* 91: 51-58.

Salinas, J. (2002). Molecular mechanisms of signal transduction in cold acclimation. In *Plant Signal Transduction*, Scheel D. and Wasternack C. (eds). Oxford University Press, Oxford, UK, pp. 116-139.

Schaller, F. and Weiler, E.W. (2002). Wound- and mechanical signalling. In *Plant Signal Transduction*, Scheel D. and Wasternack C. (eds). Oxford University Press, Oxford, UK, pp. 20-44.

Scheel, D. and Wasternack, C. (2002). Signal transduction in plants: cross-talk with the environment. In *Plant Signal Transduction*, Scheel D. and Wasternack C. (eds). Oxford University Press, Oxford, UK, pp. 1-5.

Schoonhoven, L.M., Jermy, T. and Van Loon, J.J.A. (1998). *Insect-Plant Biology, From Physiology To Evolution.* Chapman and Hall, London, UK.

Stotz, H.U., Kroymann, J. and Mitchell Olds, T. (1999). Plant-insect interactions. *Curr. Opin. Plant Biol.* 2: 268-272.

Thaler, J.S., Stout, M.J., Karban, R. and Duffey, S.S. (2001). Jasmonate-mediated induced plant resistance affects a community of herbivores. *Ecol. Entomol.* 26: 312-324.

Van Loon, J.J.A., de Boer, J.G. and Dicke, M. (2000). Parasitoid-plant mutualism: parasitoid attack of herbivore increases plant reproduction. *Entomol. Exp. Appl.* 97: 219-227.

Vet, L.E.M. and Dicke, M. (1992). Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37: 141-172.

Wäckers, F.L. and Wunderlin, R. (1999). Induction of cotton extrafloral nectar production in response to herbivory does not require a herbivore-specific elicitor. *Entomol. Exp. Appl.* 91: 149-154.

chapter

2

Signalling in plant-insect interactions:

signal transduction in direct and indirect plant defence

Marcel Dicke and Remco M.P. van Poecke

"Jargon is invented ... to prevent scientists from communicating with each other"

Terry Pratchett - The dark side of the sun

Chapter 2

SIGNALLING IN PLANT-INSECT INTERACTIONS: SIGNAL TRANSDUCTION IN DIRECT AND INDIRECT PLANT DEFENCE

Abbreviations

ABA	Abscisic acid
ACO	1-Aminocyclopropane-1-carboxylic acid oxidase
ACS	1-Aminocyclopropane-1-carboxylate synthase
CHS	Chalcon synthase
DMNT	4,8-Dimethyl-1,3(E),7-nonatriene
HMGR	3-Hydroxy-3-methylglutaryl coenzyme A reductase
HRPG	Hydroxyproline-rich glycoproteins
JA	Jasmonic acid
MeJA	Methyl jasmonate
MeSA	Methyl salicylate
DN-OPDA	dinor-oxo-phytodienoic acid
OPDA	12-oxo phytodienoic acid
PAL	Phenylalanine ammonia-lyase
PI	Proteinase inhibitor
Pin	*P*roteinase *in*hibitor gene
SA	Salicylic acid
TMTT	4,8,12-Trimethyl-1,3(E),7(E),11-tridecatetraene

---

1. Introduction

Insects make up the most speciose group of organisms that comprises more than 800,000 species (new species are still discovered at high frequency) and thus ca. 60% of all species on earth are insects. Also in numbers of individuals, insects are abundant and they occur in virtually all habitats on earth. About half of all insect species are herbivores and the majority of these are specialists that feed on one or a few related plant species. The main feeding modes of insects are chewing-biting, cell-sucking, and sap-feeding. Herbivorous insects may live on or inside the plant tissue. For instance, insects may feed externally on leaves or stem, they may live in the stem or in a leaf of the plant, or induce a gall to live in (Schoonhoven *et al.*, 1998). There are ca. 300,000 vascular plant species (Schoonhoven *et al.*, 1998) and it is unlikely for an individual plant to live without interactions with herbivorous arthropods. However, the intensity, frequency and diversity of these interactions may vary with plant species and environment.

In addition to interactions between plants and herbivorous insects, plants also have interactions with carnivorous insects that consume the herbivores. Many carnivorous insects inhabit plants (e.g. Dicke and Sabelis, 1988; Hölldobler and Wilson, 1990) and plant characteristics can influence carnivore behaviour and carnivore-herbivore interactions. In this chapter we will deal with signalling between plants and herbivorous as well as carnivorous arthropods in the context of plant defence. We will include reference to mites that, although not belonging to the class of insects, have very similar interactions with plants (Dicke *et al.*, 1998; Sabelis *et al.*, 1999).

2. Direct vs. indirect plant defence

Plants have evolved a wide range of defences to cope with the attack of herbivorous insects and mites (Karban and Baldwin, 1997; Schoonhoven *et al.*, 1998; Agrawal *et al.*, 1999). Traditionally, plant characteristics that directly affect herbivores have been studied (Karban and Baldwin, 1997; Schoonhoven *et al.*, 1998). This so-called 'direct defence' may involve physical defences (e.g., a thick cuticle, trichomes and thorns) and chemical defences (e.g., toxins, repellents and digestibility reducers). Defence chemicals can be found in all major classes of plant secondary metabolites: for instance, nitrogen-containing metabolites like alkaloids and glucosinolates, phenolics like phenylpropanoids and flavonoids, and terpenoids. Additionally, plants may use defence-related proteins. Physical and chemical factors are often combined; examples comprise toxic, deterrent and/or sticky compounds in glandular trichomes and deterrents in the epicuticular wax (Schoonhoven *et al.*, 1998).

In addition to direct defences, plants can use other mechanisms to protect themselves. Herbivores have a variety of natural enemies such as predators or parasitic wasps (parasitoids) and plants may enhance the effectiveness of these enemies and employ them as 'bodyguards'. This has been termed 'indirect defence'. This type of defence may include the provision of shelter, alternative food and chemical information, either alone or in combinations (Dicke, 1999b; Sabelis *et al*., 1999). For instance, many plants have so-called 'domatia' which are structures used as shelter by carnivores such as ants or predatory mites. These inhabitants provide protection to the plant by removing herbivores (Grostal and O'Dowd, 1994). Plants also provide floral or extrafloral nectar that carnivorous arthropods feed on and the production of these nutrient sources can be induced by herbivory (Koptur, 1992; Wäckers and Wunderlin, 1999). Finally, plants may lure carnivorous arthropods with plant volatiles produced in response to herbivore attack (Turlings and Benrey, 1998; Dicke, 1999b).

This chapter will focus on induced plant defence in response to insects and mites and the consequent interactions between plants and arthropods. The main interest will be a comparison of signal-transduction processes in direct and indirect defence.

3. Induction of direct defence

The inducibility of direct defence appears to be a general characteristic that has been demonstrated in more than 100 plant species in 34 families (Karban and Baldwin, 1997). Many types of direct defences can be induced in plants. This relates to both physical and chemical defences, but the induction of chemical defences has received most attention. Duration of the effect of induced defence ranges from a few hours to several years. The type and extent of defence varies with both the plant and herbivore species. Differences in induced defence can also be found within a plant: young plant parts often show a stronger induced defence than older parts.

An important aspect of herbivory is wounding of the plant and mechanical wounding can mimic the effect of herbivory in many induced direct defences. Therefore, mechanical damage has often been used to study the signal transduction pathway of defence induced by herbivory.

For many plant responses induced by insects the underlying mechanism is unknown (Karban and Baldwin, 1997). However, the mechanisms involved in several induced responses have been especially well studied in the last decades, such as the wound-induced production of proteinase inhibitors (PIs) in solanaceous plants, especially tomato (Ryan, 1992), and of nicotine in tobacco (Baldwin, 1999).

3.1 Proteinase inhibitors

Pioneering studies on induced direct defence were devoted to the induction of proteinase inhibitors in solanaceous plants. Herbivore feeding or mechanical wounding of potato and tomato plants result in the systemic expression of genes encoding PI proteins (Green and Ryan, 1971; Farmer and Ryan, 1990; Peña-Cortés *et al*., 1993; Doares *et al*., 1995). Proteinase inhibitors interfere with the digestive system of insects, retarding larval growth and development (Broadway *et al*., 1986). This negative effect of PIs on larval growth and development has been shown for several plant-herbivore combinations (Ryan, 1990). Elimination of PI induction can alleviate the effects on herbivores. For instance, mutant tomato plants that are impaired in a specific step in the signal-transduction leading to PI-gene induction are more susceptible to feeding by *Manduca sexta* than wild-type plants (Howe *et al.*, 1996).

Proteinase inhibitors have been found in several plant families including the Brassicaceae, Cucurbitaceae, Fabaceae, Salicaceae and Solanaceae. It has been estimated that each plant genome contains one to two hundred different PI genes, grouped in several families and inhibiting each of the four classes of proteolytic enzymes (serine, cystein, aspartic, and metallo-proteinases; Karban and Baldwin, 1997). This may be explained by the finding that herbivores can alter the set of digestive proteinases expressed, which may (temporarily?) overcome the inhibition of their digestive capabilities by the plant (Jongsma *et al.*, 1995).

Apart from being involved in direct defence, PI's may also affect indirect defence. The reduced growth rate that is caused by PI's – even when occurring temporarily – may prolong the time window during which the herbivores are exposed to their natural enemies and thus may increase mortality incurred by carnivores (Loader and Damman, 1991).

3.2 Plant secondary metabolites

Herbivory or mechanical wounding can also result in the induced production of low molecular weight secondary metabolites that may originate from all major classes of secondary metabolites (e.g. Bodnaryk, 1992; Gundlach *et al.*, 1992; Karban and Baldwin, 1997; Berenbaum and Zangerl, 1999). Genes encoding important enzymes in the production pathways of these metabolites can be induced by wounding or by treatment with plant hormones such as jasmonic acid. Examples are genes involved in the flavonoid biosynthesis like chalcone synthase (CHS; Creelman *et al.*, 1992; Gundlach *et al.*, 1992) and phenylalanine ammonia-lyase (PAL; Berger *et al.*, 1996).

A well-studied system is induced nicotine production. Natural folivory or mechanical damage inflicted to tobacco induces the *de novo* biosynthesis of the alkaloid nicotine, resulting in a four- to tenfold increase in concentration in the leaves. These concentrations are high enough to be lethal for many herbivore species (Baldwin, 1999). The increased nicotine production takes place in the roots. The key regulatory enzyme in the nicotine synthesis, putrescine *N*-methyltransferase, is upregulated at mRNA level by wounding (Hibi *et al.*, 1994). Via the xylem, nicotine is then transported to the shoots (Baldwin *et al.*, 1997; Karban and Baldwin, 1997).

The consumption of nicotine by herbivores may have a negative effect on indirect defence. For instance, mortality among *M. sexta* caterpillars from parasitoids and pathogens is lower when the herbivores have fed on a high-nicotine food source (Barbosa *et al.*, 1986; Krischik *et al.*, 1988).

3.3 Signal transduction

The mechanisms of induction of direct defences have been well studied for several systems. A few major signal-transduction routes can be distinguished. These are centred around different plant hormones such as jasmonic acid and ethylene.

3.3.1 Jasmonic acid and other oxylipins

Jasmonic acid (JA) is a product of the lipoxygenase pathway, also called the octadecanoid pathway (Sembdner and Parthier, 1993; Mueller, 1997). The lipoxygenase pathway starts with the substrate linolenic acid and results in products such as JA, traumatin and a variety of six carbon volatile compounds like hexanal, (*E*)-2-hexenal, and (*Z*)- and (*E*)-3-hexenol (Croft *et al.*, 1993; Mueller, 1997). The pathway of JA biosynthesis is probably constitutively expressed (Farmer and Ryan, 1992), but can additionally be induced by e.g. wounding (Creelman *et al.*, 1992; Peña-Cortés *et al.*, 1993), feeding by insects (Blechert *et al.*, 1995; McCloud and Baldwin, 1997), or application of microbial cell wall components (Blechert *et al.*, 1995). Methyl jasmonate (MeJA) is a volatile derivative of JA, and may function as an airborne signal molecule (Farmer and Ryan, 1990; Enyedi *et al.*, 1992; Karban *et al.*, 2000). MeJA can probably be easily converted to JA in the plant (Farmer and Ryan, 1990). Therefore, MeJA is included in this section on jasmonic acid.

Jasmonic acid is a central molecule in induced direct defence against insects in many plant species (Karban and Baldwin, 1997). The importance of jasmonic acid in wound-induced defence responses has been demonstrated by the fact that (i) exogenous application of JA or MeJA induces these defence responses, (ii) the increase of endogenous JA after wounding correlates with the induced defence responses, and (iii) inhibition of the JA production pathway also inhibits the induction of the defence responses (Baldwin *et al.*, 1997). In addition, transgenic plants and mutants have been important tools to elucidate the role of jasmonic acid in signal-transduction involved in direct plant defence (Bell *et al.*, 1995; Howe *et al.*, 1996; McConn *et al.*, 1997).

Besides jasmonic acid, several other, related, oxylipins appear to function as signaling molecules as well. These comprise e.g., 12-oxo phytodienoic acid (OPDA; Blechert *et al.*, 1995) and dinor-oxo-phytodienoic acid (DN-OPDA; Weber *et al.*, 1997). For instance, OPDA and 10,11-dihydro-OPDA have been shown to induce the same production of secondary metabolites as JA in *Eschscholtzia california* cell suspension cultures, without the conversion to JA being necessary (Blechert *et al.*, 1995). Combinations of oxylipins may result in specific plant responses and this has resulted in the designation of 'oxylipin signatures' being important in the induction of plant defences (Weber *et al.*, 1997).

3.3.2 Systemin

An 18-amino acid polypeptide called systemin has been identified in tomato as a potent inducer of PIs. So far, systemin homologues have only been found in members from the Solanaceae plant family, namely tomato, potato, black nightshade and bell pepper (Constabel *et al.*, 1998). Most research has been done on tomato.

Wounding induces the systemic accumulation of systemin in tomato by an increased expression of the gene encoding the precursor of systemin, prosystemin (McGurl *et al.*, 1992). As prosystemin mRNA is systemically produced after wounding, it is not clear whether systemin is the systemic signal, inducing its own gene expression, or that another systemic signal is involved. It has been shown however, that systemin is transported from the wound site throughout the plant within 90 min. after wounding (Narvaez-Vasquez *et al.*, 1995). Moreover, systemin induces prosystemin gene activity (Bergey *et al.*, 1996).

Application of systemin through the cut stems of tomato plants induced both the accumulation of PI proteins (Pearce *et al.*, 1991) and mRNA (Bergey *et al.*, 1996). In transgenic tomato plants with an antisense prosystemin gene, and thus a lowered systemin production, less PI protein accumulated after wounding. These transgenic tomato plants were more susceptible to feeding by *Manduca sexta* larvae (Orozco-Cardenas *et al.*, 1993). Besides the *Pin* (*P*rotein *in*hibitor) genes, several other tomato genes are induced by systemin. These include genes encoding for other defensive proteins like polyphenol oxidase (Constabel *et al.*, 1995), signal pathway-associated proteins like lipoxygenase, proteolytic enzymes and other proteins (Bergey *et al.*, 1996). These results show that systemin plays an important role in the signal transduction of wound-induced defences in tomato.

3.3.3 Ethylene

The plant hormone ethylene is produced in response to wounding (Enyedi *et al.*, 1992), herbivory (Martin *et al.*, 1988; Rieske and Raffa, 1995; Kahl *et al.*, 2000) and the application of systemin or JA in tomato cell suspensions (Felix and Boller, 1995; O'Donnell *et al.*, 1996). In tomato, both genes encoding enzymes involved in ethylene production from S-adenosyl-methionine, 1-aminocyclopropane-1-carboxylate synthase (ACS) (Yip *et al.*, 1992) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) (Barry *et al.*, 1996), are upregulated by wounding as well.

Like wounding, exogenous application of ethylene induces the production of enzymes, such as PAL, CHS, and hydroxyproline-rich glycoproteins (HRPG, involved in cell wall strengthening) mRNA. However, for HRPG it was shown that different isoforms are induced by wounding compared to ethylene application (Ecker and Davis 1987).

3.3.4 Abscisic acid

Abscisic acid is involved in several wound-induced responses, such as induced leucine aminopeptidase (Chao *et al.*, 1999) or PI's (Peña-Cortés *et al.*, 1989, but see Chao *et al.*, 1999 and Birkenmeier and Ryan, 1998). In many other wound-induced responses ABA does not seem to play a role (e.g. Laudert and Weiler, 1998). In several induced defences the role of abscisic acid is not clear and its role in e.g. the induction of proteinase inhibitors is under debate (Chao *et al.*, 1999 and Birkenmeier and Ryan, 1998). How exactly ABA is involved in the signal transduction pathways is not clear. For example, water stress promotes an increase in endogenous ABA levels with a factor 8 to 10, but this does not lead to *Pin2* gene expression (Peña-Cortés *et al.*, 1989). The induction of water stress-responsive genes does not require *de novo* synthesis of proteins, whereas the induction of *Pin2* by JA does. Recent data suggest that ABA is not a primary signal for *Pin2* gene induction, but that it may modulate the responses to other signals (Birkenmeier and Ryan, 1998; Chao *et al.*, 1999).

3.3.5 Electrical signals

Electrical signals may play a role in the systemic induction of PIs in tomato (Wildon *et al.*, 1992), but so far it remains unclear how these signals interact with chemical signals.

3.3.6 Insect damage versus mechanical damage: herbivore elicitors

As mentioned before, most research has been done on mechanical damage. Several publications report differences between herbivory- and wound-induced responses (e.g. Baldwin, 1990; Hartley and Lawton, 1991; Stout *et al.*, 1994; McCloud and Baldwin, 1997; Felton and Eichenseer, 1999). Of course, herbivory is more than mechanical wounding and plants may use for instance oral secretions from herbivores to differentiate between mechanical wounding and herbivory (Lin *et al.*, 1990; Hartley and Lawton, 1991; Korth and Dixon, 1997; McCloud and Baldwin, 1997; Felton and Eichenseer, 1999).

Differences between herbivory- and wound-induced responses have been found in *Pin2* gene expression in potato (Korth and Dixon, 1997) and in nicotine accumulation in tobacco (McCloud and Baldwin, 1997). Induction of the *Pin2* gene in potato appeared to occur faster by feeding of *Manduca sexta* larvae than induction by artificial wounding. Similar results were obtained with the 3-hydroxy-3-methylglutaryl-coenzymeA (HMGR) reductase gene family from potato. HMGR is the first enzyme in the mevalonic acid-derived terpenoid biosynthetic pathway. The early induction of both *Pin2* and HMGR mRNA could be mimicked by application of *Manduca sexta* larvae regurgitant on artificially wounded leaves and is probably caused by an insect-derived, heat-stable elicitor from the regurgitant. These results indicate that the signalling pathways of herbivory-induced and wound-induced plant defences may be at least partially different (Korth and Dixon, 1997). In tobacco, herbivory by *Manduca sexta* larvae or application of their regurgitant decrease the induction of nicotine production compared to mechanical damage, even though JA induction was increased (McCloud and Baldwin, 1997). A similar effect was recorded for the induction of trypsin inhibitor by *M. sexta* in tomato (Jongsma *et al.*, 1994). It seems that *Manduca sexta* larvae are able to suppress induced defences by influencing the signal pathway (McCloud and Baldwin, 1997).

A component of the saliva of *Helicoverpa zea* caterpillars, the enzyme glucose oxidase, has been found to induce the salicylate pathway in soybean, tobacco and cotton, resulting in systemic acquired resistance to *Pseudomonas syringae*. This

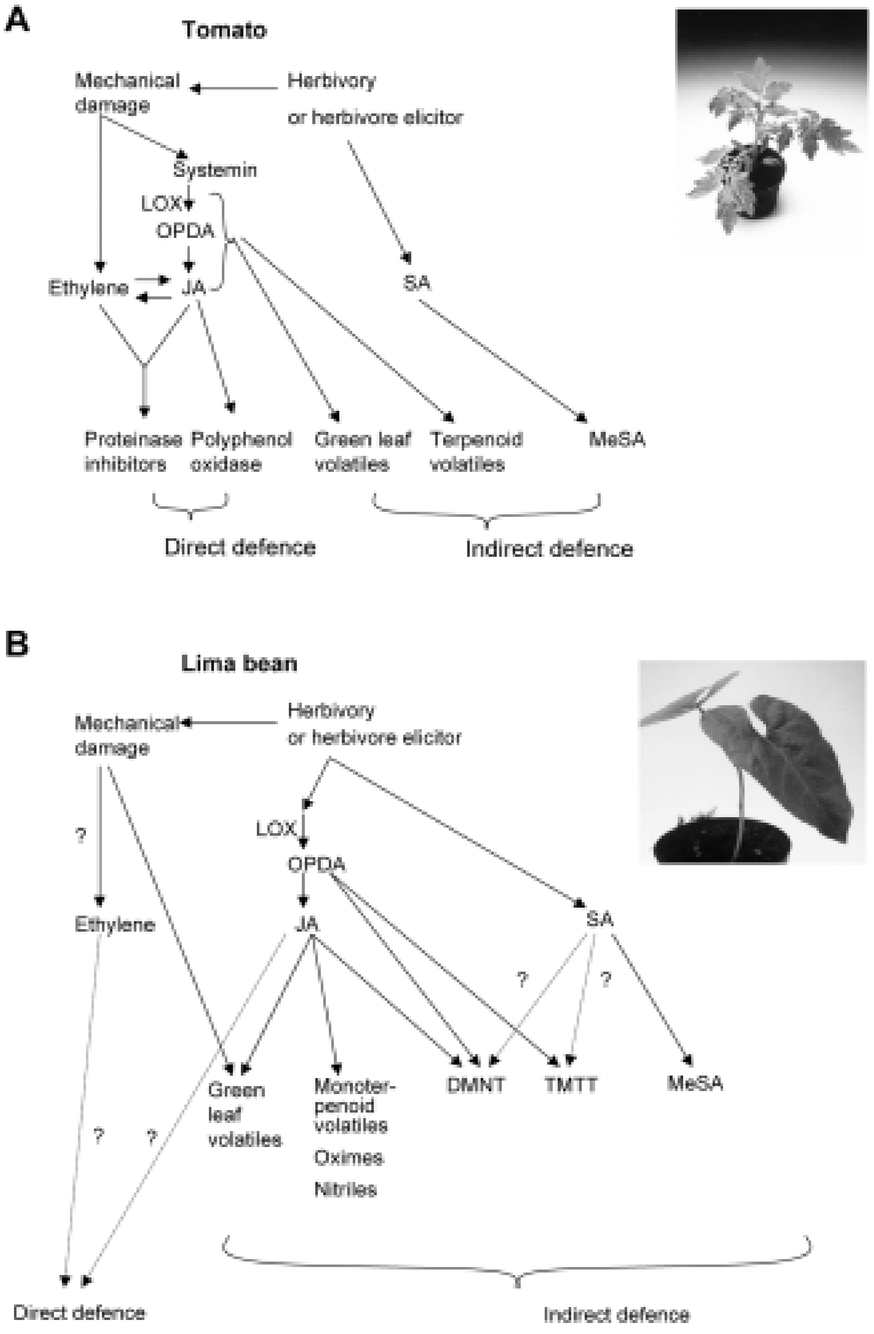


Figure 1. Signal transduction pathways involved in defences induced by herbivory versus mechanical damage:
(a) in tomato, where herbivory and mechanical damage have many similar effects (based on Farmer and Ryan, 1992; O'Donnell *et al.*, 1996; Dicke *et al.*, 1998; and Boland *et al.*, 1999)
(b) in Lima bean, where herbivory and mechanical damage have rather different effects (based on Dicke *et al.*, 1990b; Boland *et al.*, 1999; Dicke *et al.*, 1999; Arimura *et al.*, 2000; and Ozawa *et al.*, 2000).

effect was not found in response to mechanical damage (Eichenseer *et al.*, 1999; Felton and Eichenseer, 1999).

Although there are differences in plant responses to artificial wounding and herbivory, there are still enough indications that the signal pathways invoked by mechanical wounding or insect feeding share at least some components (Figure 1). Knowledge on the signal transduction pathway of wound-induced plant defences will help us unravel the signal transduction pathway of defences induced by herbivory.

3.3.7 Cross-talk and interactions among signal-transduction pathways

The emerging picture of signal-transduction in direct defence is that different signal-transduction pathways interact. For instance, salicylic acid (SA), which can be induced by pathogen attack (see Whitham and Dinesh-Kumar, 2002), interferes with jasmonic acid-mediated responses involved in defence against herbivorous arthropods. SA blocks the biosynthesis of jasmonic acid and subsequent induction of gene expression (Peña-Cortés *et al.*, 1993; Doares *et al.*, 1995) and consequently affects the defence of plants against herbivores (Thaler *et al.*, 1999). For instance, treatment of plants with an SA mimic (benzothiodiazole-7-carbothioic acid *S*-methyl ester, BTH) alleviates the effect of JA-treatment of tomato plants on the induction of polyphenol oxidase activity and herbivory by caterpillars of *Spodoptera exigua* (Thaler *et al.*, 1999). Vice versa, JA can inhibit the effect of salicylic acid (Sano and Ohashi, 1995) or the synthetic mimic BTH (Thaler *et al.*, 1999). For instance, JA treatment of tomato plants eliminates the induction of the *PR-4* gene and reduces the protection of BTH-treated tomato plants against *Pseudomonas syringae* pv tomato (Thaler *et al.*, 1999). In other cases, cross-induction has been reported. For instance, the spider mite *Tetranychus urticae* and the fungus *Verticillium dahliae* induced resistance against spider mites and the fungus in cotton (Karban *et al.*, 1987). How this is related to the induction of JA and/or SA remains unclear.

JA and ethylene act in concert in the induction of *Pin* gene expression in tomato (Figure 1A). These two plant hormones influence each other's level in wounded plants and are needed together for induction of PI's in tomato (O'Donnell *et al.*, 1996). Ethylene alone does not induce the *Pin* genes in tomato (Ryan, 1974), but inhibition of ethylene action inhibits the induction of *Pin2* in tomato by wounding, systemin, JA, and oligogalacturonide fragments. These results suggest that ethylene action is downstream from JA in the wound response pathway. However, when ethylene action is blocked, the induction of endogenous JA levels by wounding is reduced. It appears that ethylene and JA induce each other's production (O'Donnell *et al.*, 1996).

Signal-transduction pathways can be specific for the plant species and the tissue within a plant. Tomato plants respond to cell-wall derived oligosaccharides or chitosan with *de novo* synthesis of JA which results in the accumulation of PI's (Doares *et al.*, 1995; O'Donnell *et al.*, 1996). In contrast, in *Arabidopsis* treatment with chitosan does not result in elevated JA levels, which is mediated by ethylene-dependent negative effects on JA effectiveness. This ethylene-dependent blocking of JA-mediated effects in *Arabidopsis* does not occur in systemic tissues. As a consequence, different responses are induced in local and systemic tissues (Rojo *et al.*, 1999). Also in nicotine induction in tobacco, ethylene and JA have opposite rather than synergistic effects (Kahl *et al.*, 2000). Thus, specifics of the interaction among different signal-transduction pathways seem to be dependent on the plant species. Obviously, more studies are needed to obtain a better understanding of general effects and exceptions.

4. Induction of indirect defence

Research on the induction of indirect defence has started in the mid 1980's (Sabelis and Dicke, 1985; Dicke and Sabelis, 1988; Dicke *et al.*, 1990b; Turlings *et al.*, 1990; Mattiacci *et al.*, 1994; Turlings *et al.*, 1995; Takabayashi and Dicke, 1996) and has mainly concentrated on the induction of carnivore-attracting plant volatiles. Herbivory by arthropods results in the emission of a blend of volatiles that attracts the enemies of herbivorous arthropods. This has been studied in depth for plant – spider mite – predatory mite interactions (e.g. Dicke *et al.*, 1998) and plant – caterpillar – parasitoid interactions (e.g. Turlings *et al.*, 1995).

The induction of plant volatiles has been recorded for more than 23 plant species from 13 families (Dicke, 1999c) and it seems that it is a common response of plants to herbivory. In all plant species investigated the ability has been found. Among the plant families investigated are e.g., the Fabaceae, Brassicaceae, Cucurbitaceae, Poaceae, Malvaceae, Solanaceae, Rosaceae, and the Asteraceae. Two types of plant response can be distinguished (Dicke, 1999c).

(1) In response to herbivory the plant produces a blend that is dominated by novel compounds that are not emitted by intact or mechanically damaged plants. This type of response can be found in e.g. Lima bean (Figure 1B), cucumber, maize, and gerbera (Dicke *et al.*, 1990b; Turlings *et al.*, 1990; Takabayashi *et al.*, 1994b; Krips *et al.*, 1999).

(2) In response to herbivory the plant produces a blend that is qualitatively similar to the blend emitted by intact or mechanically damaged plants. In the latter case, the emission rate from herbivore-damaged plants is much higher than from mechanically damaged or undamaged plants and it continues much longer after termination of the damage (e.g. Bolter *et al.*, 1997). This type of response has been recorded for e.g., cabbage, cotton, tomato (Figure 1A) and potato (Agelopoulos and Keller, 1994; Mattiacci *et al.*, 1994; McCall *et al.*, 1994; Bolter *et al.*, 1997; Dicke *et al.*, 1998).

In both types of responses, de novo biosynthesis of volatiles has been reported (Donath and Boland, 1994; Paré and Tumlinson, 1997) and thus plants invest in the production of volatiles rather than passively emitting the contents of damaged cells. Moreover, herbivore-induced plant volatiles are emitted systemically (Dicke et al., 1990a; Turlings and Tumlinson, 1992; Dicke et al., 1993; Röse et al., 1996) which further supports the conclusion that the emission of volatiles is an active response rather than a passive release of cell contents.

The herbivore species that have been shown to induce plant volatiles belong to 27 species in 13 families of insects and mites. These herbivores include folivores (chewing-biting and cell-sucking species), sap-feeding insects, and species that feed in the plant such as leaf miners and stem borers (Dicke, 1999c). Plants may even induce volatiles in response to oviposition by a herbivore (Meiners and Hilker, 2000). The emitted blend varies largely among plant species in a qualitative sense. In addition, the blend also varies among plants of the same species that are damaged by different herbivores. However, this variation is much more subtle and usually relates to quantitative variation in the blend composition, i.e. the blends are composed of the same constituents but the relative contribution of different constituents to the blend varies (see Dicke, 1999a for review). Sometimes, a qualitative difference between blends of plants emitted by different herbivores has been recorded, e.g. for faba beans infested with different aphid species (Du et al., 1998). Furthermore, the blend may be affected by herbivore instar, as was recorded for maize plants damaged by caterpillars (Takabayashi et al., 1995).

In addition, abiotic factors may affect the emission of plant volatiles in a quantitative and qualitative sense (e.g. Blaakmeer et al., 1994; Takabayashi et al., 1994a), but little efforts have been made to investigate the effect of abiotic conditions on the emission of herbivore-induced plant volatiles.

In conclusion, herbivory results in a change in the emission of volatiles and the composition of the emitted blend varies with biotic and abiotic factors.

4.1 Identity of herbivore-induced plant volatiles

The major volatiles emitted by plants, either constitutively or induced, belong to several classes that are produced through distinct biosynthetic pathways: e.g. fatty acid-derivatives produced through the lipoxygenase pathway, terpenoids produced through the isoprenoid pathway and phenolics produced through the shikimic acid pathway.

The fatty acid-derived volatiles comprise C6-aldehydes, C6-alcohols and their esters such as (Z)-3-hexen-1-yl acetate. They are common plant volatiles (Hatanaka et al., 1987) and are often referred to as green-leaf volatiles (Visser and Avé, 1978). They are emitted in response to artificial damage as well as herbivory (e.g., Dicke et al., 1990b; Turlings et al., 1990; Mattiacci et al., 1994). These compounds can be perceived by parasitoid chemoreceptors (Van Loon and Dicke, 2000) and some parasitoid species are attracted by these green leaf odors (Whitman and Eller, 1992).

Terpenoids comprise the largest and most diverse chemical group in plants. They can be produced through the mevalonic acid pathway (Gershenzon and Croteau, 1991) or through the 1-deoxy-D-xylulose-5-phosphate pathway (Lichtenthaler, 1999) and many of them are well known for their toxic effects on herbivores (Gershenzon and Croteau, 1991). Among the herbivore-induced terpenoids there are two compounds that are especially noteworthy, i.e. the homoterpenes 4,8-dimethyl-1,3(*E*),7-nonatriene and 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene. These terpenoids have been recorded from many plant species after the infliction of herbivory or treatment with elicitors (Dicke *et al.*, 1990b; Turlings *et al.*, 1990;

Boland *et al*., 1992; Dicke, 1994). Herbivory leads to the induction of (3S)-(*E*)-nerolidol synthase in Lima bean, cucumber and corn and subsequently to the formation of 4,8-dimethyl-1,3(*E*),7-nonatriene (Bouwmeester *et al*., 1999; Degenhardt and Gershenzon, 2000), which is a known attractant of the carnivorous mite *P. persimilis* (Dicke *et al*., 1990b).

Phenolics are produced through the shikimic acid pathway. Among the phenolics emitted in response to herbivory are indole and methyl salicylate. Indole is an intermediate product of tryptophan biosynthesis. It plays an important role in direct defence as a component of indole alkaloids and indole glucosinolates (e.g. Bodnaryk, 1992). It has also been recorded among herbivore-induced plant volatiles, e.g. from maize, cowpea, soybean, cotton, gerbera or Lima bean (Turlings *et al*., 1990; McCall *et al*., 1994; Takabayashi *et al*., 1995; Dicke *et al*., 1999; Krips *et al*., 1999). Methyl salicylate is the volatile methyl ester of the plant hormone salicylic acid. It has been recorded in several plant species, such as Lima bean, apple, and pear (Dicke *et al*., 1990b; Takabayashi *et al*., 1991; Scutareanu *et al*., 1997).

Nitrogen-containing compounds such as nitriles and oximes are commonly reported from herbivore-damaged plants. In crucifers these compounds can be degradation products of glucosinolates (Mattiacci *et al*., 1994; Geervliet *et al*., 1997), whereas in other plant species such as cucumber, Lima bean or gerbera (Dicke *et al*., 1990b; Takabayashi *et al*., 1994b; Krips *et al*., 1999), they may be derived from amino acids (Kaiser, 1993).

4.2 Importance of herbivore-induced plant volatiles to carnivorous arthropods

For natural enemies, the most reliable information on the presence of their herbivorous victim of course originates from the herbivore itself. However, a herbivorous arthropod is only a small component of the environment with a small biomass. Moreover, herbivores have been under natural selection to avoid being detectable by their natural enemies (Vet and Dicke, 1992). Behavioural studies show that herbivorous arthropods or their faeces are not very attractive to carnivorous enemies (e.g. Sabelis *et al*., 1984a; Turlings *et al*., 1991; Steinberg *et al*., 1993; Geervliet *et al*., 1994) and a chemical analysis showed that hardly any volatiles can be detected from *Spodoptera exigua* larvae and faeces (Turlings *et al*., 1991). Plants represent a much larger biomass and detection of herbivores by their natural enemies can benefit plants (Van Loon *et al*., 2000a). For example, arthropod carnivores such as predatory mites are well known to exterminate local populations of their herbivorous prey (Sabelis and Dicke, 1985).

It is well established that herbivore-induced plant volatiles play an important role in the attraction of carnivores. For instance, the predatory mite *Phytoseiulus persimilis* (Sabelis *et al*., 1984a), and the parasitoid wasps *Cotesia marginiventris* (Turlings *et al*., 1991), *Cotesia glomerata* (Steinberg *et al*., 1993), *Cotesia rubecula* (Geervliet *et al*., 1994) and *Microplitis croceipes* (McCall *et al*., 1993) all prefer odours from the plant-host complex over those from faeces of their herbivorous host/prey. The blend of volatiles can be specific for the herbivore species that induced it. Many arthropod predators and parasitoids have been shown to discriminate between plants infested with different herbivore species and also chemical differences have been reported (see Dicke, 1999a for review). Thus, the induced plant volatiles are important cues for carnivores to locate their herbivorous victims. The discrimination among induced blends of plant odours may need to be learned or can be dependent on the physiological condition (e.g. starvation level) of the carnivore (Dicke, 1999a).

Because herbivore-induced carnivore-attractants are emitted at the site of damage as well as systemically from undamaged leaves (Dicke *et al.*, 1990a; Turlings and Tumlinson, 1992; Röse *et al.*, 1996), the odour source is much larger than the herbivore, which increases the detectability of the herbivore to its natural enemies.

4.3 Response by carnivorous arthropods and benefits to plants

Herbivore-induced plant volatiles are very important for carnivorous arthropods and the carnivores can use them during several foraging decisions. This has been shown for a large number of carnivorous arthropods, including e.g. parasitic wasps (Turlings *et al.*, 1990; Geervliet *et al.*, 1994), predatory bugs (Scutareanu *et al.*, 1997; Van Loon *et al.*, 2000b), and predatory mites (Sabelis and Van de Baan, 1983; Dicke *et al.*, 1998). Parasitoids and predators can be attracted from a distance and this has been demonstrated in various setups, including olfactometers (Sabelis and Van de Baan, 1983), windtunnels (Steinberg *et al.*, 1993), semi-field setups (Wiskerke and Vet, 1994; Janssen, 1999) and field tests (De Moraes *et al.*, 1998). In addition, the volatiles can also mediate arrestment in a herbivore patch (Sabelis *et al.*, 1984b) or the suppression of long-range dispersal (Sabelis and Afman, 1994).

Plants can greatly benefit from the attraction of carnivorous arthropods. Carnivores such as predatory mites or predatory bugs eliminate the herbivores by predation. They can exterminate local prey populations and thus relieve the plant of their herbivores. However, in the case of parasitoids this benefit to the plant is not self-evident (Van Loon *et al.*, 2000a). After parasitization the herbivore is not killed but in most cases continues to feed from the plant, sometimes at a larger rate than when not being parasitized (Harvey, 2000). The latter may be dependent on the number of parasitoid eggs deposited in the herbivore. In order to establish whether plants benefit from parasitoids, one should investigate the effect of parasitization on plant fitness e.g. in terms of seed production. This has recently been done for *Arabidopsis thaliana* plants infested with *Pieris rapae* caterpillars and the solitary parasitoid *Cotesia rubecula*. This study showed that parasitization of the caterpillars resulted in a large reduction in fitness loss compared to plants infested with unparasitized caterpillars (Van Loon *et al.*, 2000a). Whether this is a general phenomenon should become clear from the investigation of other plant-herbivore-parasitoid systems.

4.4 Herbivore-induced plant volatiles and responses by herbivores

After emission of the induced volatiles, the plant is no longer in control over who exploits the information. For example, herbivores that forage for food may exploit the information to find a host plant (Figure 2). To herbivores the volatiles

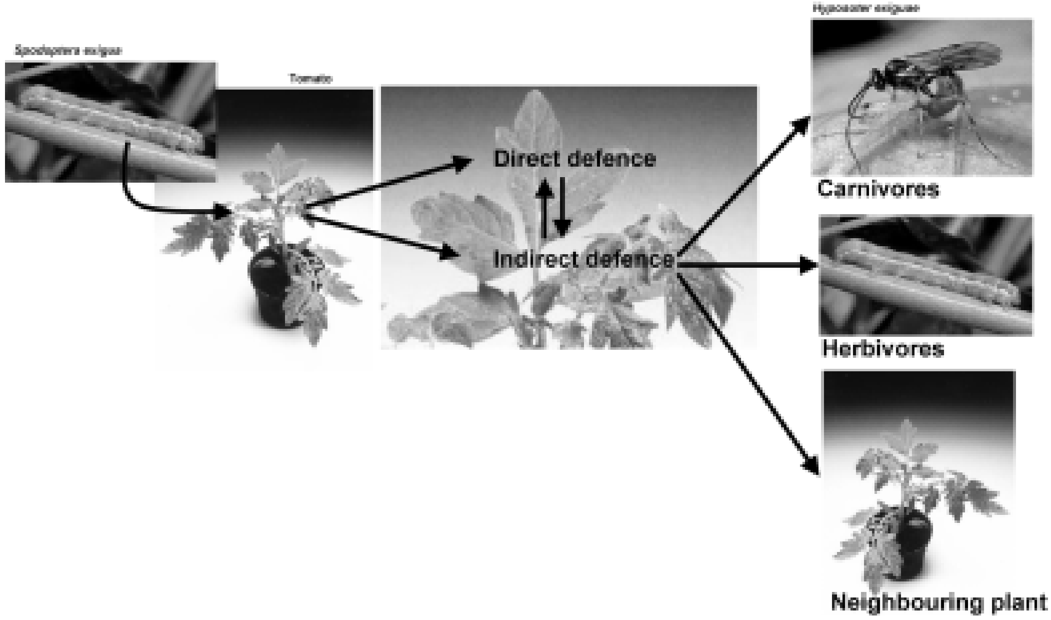


Figure 2. Herbivore-induced defences and effects on organisms at three different trophic levels, as exemplified for a tritrophic system consisting of tomato, *Spodoptera exigua* caterpillars, and the parasitoid *Hyposoter exigua*. See text for specific information. Based on data from Farmer and Ryan, 1992; Bruin *et al.*, 1995; Shonle and Bergelson, 1995; Howe *et al.*, 1996; Dicke *et al.*, 1998; Thaler, 1999; Thaler *et al.*, 1999; and Arimura *et al.*, 2000.

may represent information on (a) the presence of a host plant, (b) the presence of competitors and (c) the presence of a potentially enemy-dense space and herbivores may exploit this information. However, these are conflicting types of information and one may predict that the responses of herbivores are dependent on external and internal conditions. For instance, the information may lead to a different response in a starved herbivore than in a satiated herbivore and the response may also depend on other information, e.g. on the presence of carnivorous enemies in the environment. Indeed, different responses have been recorded for herbivores (for reviews see Dicke and Vet, 1999; Sabelis *et al.*, 1999; Dicke and Van Loon, 2000). For instance, the spider mite *Tetranychus urticae* is attracted to volatiles from undamaged Lima bean plants and to volatiles from a combination of volatiles from undamaged leaves and from spider-mite infested leaves. In contrast, when only volatiles from spider-mite infested leaves are offered, the spider mites are repelled (Dicke, 1986). Alternatively herbivores may be attracted to plants infested with heterospecific herbivores because the heterospecifics provide protection from natural enemies (Shiojiri *et al.*, 2000).

For herbivores that feed on the volatile-emitting plant, the volatiles may also have a direct negative effect. For instance, the green-leaf volatiles have a negative effect on the rate of population increase of aphids (Hildebrand *et al.*, 1993). This resembles the negative effect of these compounds on bacterial proliferation (Croft *et al.*, 1993). Thus, these induced compounds may play a role in both direct and indirect defence. Most likely other induced volatiles such as terpenes can also interfere directly with herbivore performance and thus play a dual role.

4.5 Herbivore-induced plant volatiles and effects on neighbouring plants

The information emitted by infested plants may potentially also affect downwind uninfested neighbours (Figure 2). These neighbouring plants may exploit the information to initiate defences. After all, after the upwind neighbour has been overexploited by the herbivores they may reach the downwind neighbour on wind currents just as the information did. Information transfer between herbivore-infested and uninfested plants has been investigated since the mid 1980s (reviewed in Bruin et al., 1995; Shonle and Bergelson, 1995; Karban and Baldwin, 1997; Dicke and Vet, 1999). Evidence in favour of the exploitation of the information by downwind plants is increasing and recently some exciting new data have been published (Arimura et al., 2000; Karban et al., 2000).

4.6 Signal transduction

Carnivorous arthropods can discriminate between volatiles emitted by herbivore-damaged and mechanically-damaged plants (for reviews see e.g. Vet and Dicke, 1992; Dicke, 1999c). This is true both for plants that produce novel compounds in response to herbivory and for plants that produce the same compounds in response to herbivory and to mechanical damage. In the latter case the volatiles are emitted in larger amounts and especially during a longer period of time in response to herbivory than in response to wounding (e.g. Bolter *et al.*, 1997). This has stimulated the search for herbivore elicitors that enable the plant to discriminate between wounding and herbivory. In addition, the extensive knowledge on signal-transduction pathways in direct defence has been utilized to investigate their involvement in induced indirect defence.

4.6.1 Herbivore elicitors

As indicated above, mechanical damage can usually not effectively mimic herbivory in the induction of indirect defence. This can be explained by the role of herbivore elicitors. The application of oral secretions of herbivores onto mechanical damage can result in similar effects on volatile induction as herbivory itself (Turlings *et al.*, 1990; Mattiacci *et al.*, 1994). The search for active components of the oral secretions has yielded two compounds: a β-glucosidase from *Pieris brassicae* caterpillars, which induces volatiles in cabbage, maize and bean plants (Hopke *et al.*, 1994; Mattiacci *et al.*, 1995), and the fatty acid-amino acid conjugate N-(17-hydroxylinolenoyl)-L-glutamine called volicitin, which induces volatiles in maize (Alborn *et al.*, 1997). In both cases, the application of the elicitor is a good mimic of herbivory, but it remains unknown what the exact effects of the elicitors are. Because glycosidically bound forms of the volatiles emitted have not been recorded, the glucosidase may release an internal elicitor. Volicitin, which has the fatty acid moiety 17-hydroxylinolenic acid, most likely activates the octadecanoid pathway (Alborn *et al.*, 1997). This pathway is known to be involved in the induction of carnivore-attracting volatiles (Hopke *et al.*, 1994; Boland *et al.*, 1995; Dicke *et al.*, 1999; Gols *et al.*, 1999).

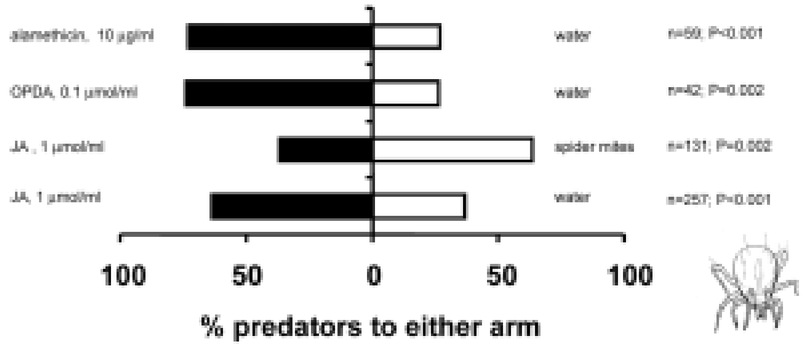
In some cases, mechanical damage can mimic herbivory in the induction of indirect defence. For example, caterpillar damage to cotton (*Gossypium herbaceum*) plants had the same effects on the induction of extrafloral nectar production as either mechanical damage in combination with caterpillar oral secretion or mechanical damaged plus water treatment (Wäckers and Wunderlin, 1999). But in general, indirect defence induced by herbivores is different from the response to mechanical damage (e.g. Takabayashi and Dicke, 1996; Dicke, 1999c; Sabelis *et al.*, 1999). An intriguing question is why herbivore elicitors appear to be more important in the induction of indirect defence than in the induction of direct defence. Is this difference an artefact, resulting from the use of different model plants? After all, the model plants of research on induced direct defence such as potato and tomato produce a similar odour blend in response to mechanical damage and herbivory, albeit that the effect of herbivory lasts longer (Bolter *et al.*, 1997; Dicke *et al.*, 1998). Or is this the result of the interaction with carnivorous arthropods that base their foraging 'decisions' upon the information provided by the plant and therefore select for the emission of more specific signals in the plant?

4.6.2 Systemic elicitor

The induction of plant volatiles occurs systemically throughout the plant (Dicke *et al.*, 1990a; Turlings and Tumlinson, 1992; Röse *et al.*, 1996). An active compound has been extracted from spider mite-infested Lima bean plants, and when applied to an uninfested plant results in attraction of carnivorous mites (Dicke *et al.*, 1993). So far, the identity of the systemic elicitor(s) has not been elucidated.

4.6.3 Jasmonic acid and octadecanoid pathway

Treatment of Lima bean, maize, and gerbera plants with JA resulted in the emission of volatiles that are also induced by herbivory (Hopke *et al.*, 1994; Boland *et al.*, 1995; Dicke *et al.*, 1999; Gols *et al.*, 1999). It is noteworthy that treatment with JA resulted in the induction of volatiles from different biosynthetic pathways in the same plant, such as the lipoxygenase pathway and the isoprenoid pathway. The volatile blend emitted by Lima bean after JA treatment is similar but not identical to the blend emitted by *T. urticae* infested plants. For instance, JA did not induce methyl salicylate or the homoterpene 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene, while it induced two methyloximes in larger amounts than did spider mite damage. This difference in odour blend results in differential behaviour of the carnivorous arthropods (Figure 3). The carnivorous mite *P. persimilis* was attracted to JA-treated plants, but in a two choice situation preferred the volatiles from spider-mite damaged plants (Dicke *et al.*, 1999). In other plants (cucumber, tobacco, and *Arabidopsis thaliana*) MeJA induced the activity of two important enzymes in the green-leaf volatile production pathway, i.e. lipoxygenase (LOX) and hydroperoxide lyase (HPL), resulting in the increased emission of green leaf volatiles by plants (Avdiushko *et*



Fihvsf 4. Attraction of predatory mite *Phytoseiulus persimilis* to Lima bean plants infested with herbivorous spider mites (*Tetranychus urticae*) or treated wiht JA, OPDA, or the peptaibol alamethicin. Experiments were carried out in a Y-tube olfactometer. For details of experimental setup, see Dicke *et al.*, 1999. Data based on Dicke *et al.*, 1999 and M. Dicke and H. Dijkman, unpublished data.

al., 1995). Treatment of plants with JA results in attraction of predators and parasitoids under laboratory and field conditions (Hopke *et al.*, 1994; Boland *et al.*, 1995; Dicke *et al.*, 1999; Gols *et al.*, 1999; Thaler, 1999). For instance, in field-grown tomato plants JA-treatment resulted in a larger number of parasitoids (*Hyposoter exiguae*) and in a higher parasitisation percentage of *Spodoptera exigua* caterpillars (Thaler, 1999). These results indicate an important role for JA as an endogenous signal molecule involved in induced production of volatiles. Indeed, both *Manducta sexta* herbivory and regurgitant treatment on mechanically damaged leaves resulted in an increase of endogenous JA levels in tobacco (McCloud and Baldwin, 1997). Although JA is induced by herbivory, its methyl ester MeJA has never been recorded from herbivore-infested plants.

Several intermediates from the octadecanoid pathway can induce plant volatiles. For instance, OPDA induces the two homoterpenes 4,8-dimethyl-1,3(E),7-nonatriene and 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene in Lima bean (Boland *et al.*, 1999) and OPDA treatment results in attraction of the predatory mite *P. persimilis* (Figure 3). Nonetheless, JA appears to be the most powerful member of the octadecanoids in terms of induction of Lima bean volatiles. A combination of octadecanoids may be responsible for the total induction pattern as recorded in response to spider mite damage. However, no herbivory-related elicitors have been found yet that induce methyl salicylate, a constituent of spider-mite induced Lima bean volatiles that attracts predatory mites (Dicke *et al.*, 1990b).

Other compounds, such as conjugates of 1-oxo-indan-4-carboxylic acid, appear to have a strong inducing power as well. Coronatin (the coronamic acid conjugate of 1-oxo-indan-4-carboxylic acid) is the phytotoxin of certain *Pseudomonas syringae* pathovars and this compound induces a similar blend in Lima bean as JA, but in addition also induces the C16 homoterpene 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene that is not induced by JA (Boland *et al.*, 1995). A synthetic analogue such as 1-oxo-indanoyl-isoleucine (IN-Leu) induced a similar volatile blend in Lima bean as JA did (Boland *et al.*, 1999). Most work on the effect of octadecanoids and their analogues has been done on Lima bean plants. To what extent these results apply to other plants remains unclear. The situation seems to be different in the monocotyledon maize. For this plant the differential effects of octadecanoids do not seem to apply (Boland *et al*., 1999).

Whether JA plays a role in other induced indirect defences as well, such as e.g. extrafloral nectar induction, has not been investigated.

4.6.4 Salicylic acid (SA)

A remarkable compound emitted by several plant species in response to herbivory is methyl salicylate. This compound is induced in e.g. spider-mite infested Lima bean and tomato plants (Dicke *et al*., 1990b; Dicke *et al*., 1998), in psyllid-infested pear plants (Scutareanu *et al*., 1997) and in Colorado potato beetle-infested potato plants (Bolter *et al*., 1997). It has also been recorded from tobacco mosaic virus-infested tobacco plants and was suggested to be a way of disposing of salicylic acid (SA) formed in infested plants (Shulaev *et al*., 1997). In cotton, herbivory by caterpillars of *Helicoverpa zea* leads to increased levels of SA, but no effect of SA induction has been found on the performance of the caterpillars (Bi *et al*., 1997). In an analysis of headspace volatiles of *H. zea*-infested cotton plants, an emission of methyl salicylate has not been reported (Turlings *et al*., 1993). The application of gaseous MeSA to Lima bean plants was reported to induce the emission of the two homoterpenes 4,8-dimethyl-1,3(E),7-nonatriene and 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene (Ozawa *et al*., 2000). It will be important to investigate the effect of SA production on indirect defence of plants, because SA is known to inhibit the effect of JA in direct defence. JA is important in the induction of plant volatiles and SA may inhibit this (Engelberth *et al*., 2000), but possibly the production of SA and the induction of plant volatiles by JA are spatially and/or temporally separated which would reduce the possiblity of interference (see below in section 4.6.7 ‘Cross talk’). If SA is induced by herbivory, its methylation and emission as MeSA is a way of avoiding the accumulation of SA in response to herbivory or possibly in response to microorganisms transmitted to the plant by the herbivore during feeding.

4.6.5 Ethylene

The induction of ethylene emission in response to herbivory or elicitors has been reported for several plants such as tobacco (Kahl *et al*., 2000) and Lima bean (Piel *et al*., 1997). To our knowledge, there is no evidence that ethylene induces plant volatiles. However, the role of ethylene in induced indirect defence has received little attention to date. In tobacco, ethylene treatment did not induce the emission of (-)-*cis*-α-bergamotene or linalool (Kahl *et al*., 2000), two compounds induced by *Manduca sexta* caterpillar feeding.

4.6.6. Other elicitors

Several other elicitors have been reported to induce plant volatiles. Among these are e.g. cellulysin and alamethicin, a mixture of the peptaibols, that are produced by the fungus *Trichoderma viride* (Engelberth *et al*., 2000). The peptaibols (oligopeptides) act as ion-channel forming compounds and are considered to mediate a very early step in the induction. In Lima bean they induce the production of a short peak of jasmonic acid and of salicylic acid. The latter phytohormone is produced at a high level for a long period of time. It is interesting to see that administration of the peptaibol alamethicin results both in a high level of SA in the plant and in the emission of MeSA. In addition to MeSA, the only other compounds emitted are the two homoterpenes 4,8-dimethyl-1,3(E),7-nonatriene and 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene. This indicates that the SA inhibits the octadecanoid pathway beyond OPDA (Engelberth *et al.*, 2000), as this octadecanoid intermediate induces the production of the two homoterpenes (Boland *et al.*, 1999).

It appears that the blends of volatiles induced by these elicitors affect the behaviour of carnivorous arthropods: the predatory mite *P. persimilis* is attracted to peptaibol-treated Lima bean plants (Figure 3). This can be explained by the observation that two of the three volatiles emitted in response to alamethicin treatment, i.e. MeSA and the homoterpene 4,8-dimethyl-1,3(E),7-nonatriene, are known to attract this predatory mite (Dicke *et al.*, 1990b). These volatiles are also induced by feeding damage of the spider mite *T. urticae*, on which this predatory mite preys.

4.6.7 Cross-talk

Research on signal transduction in induced indirect defence has a shorter history than that in induced direct defence. However, the few studies that have addressed it, indicate that this will be an essential subject for future investigations. The major pathway involved in indirect defence appears to be the octadecanoid pathway. From studies on direct defense it is well-known that the jasmonate and salicylate pathways interfere and methyl salicylate is induced in several plants by herbivory (see above). Different types of herbivores have different effects on volatile induction and the relative induction of the jasmonate and salicylate pathways may play a role. In Lima bean, spider mite feeding induces considerable emission of MeSA (Dicke *et al.*, 1990b; Ozawa *et al.*, 2000), while caterpillar feeding damage does not (Ozawa *et al.*, 2000). Application of JA and MeSA in different combinations also resulted in differences in volatile induction in Lima bean. Treatments that included both JA and MeSA applications induced compounds that are also induced by spider mite feeding, while application of JA exclusively resulted in the emission of volatiles that are also emitted in response to caterpillar feeding (Ozawa *et al.*, 2000). The possible induction of SA by spider mite feeding is supported by gene expression studies that show that PR-4 gene expression is induced by spider mite feeding and MeSA treatement, but not by caterpillar feeding or JA treatment. If herbivores, like pathogens, produce ion-channel forming compounds that induce JA and SA in plants, then the relative timing and induced levels of these phytohormones may determine the volatile profile emitted (Engelberth et al., 2000; Ozawa et al., 2000). This may be an important determinator of blend composition, that is known to vary with herbivore species or instar that feeds on the plant (Dicke, 1999a). In this context, it will be important to investigate the effects of pathogens on induced indirect defence against herbivores. So far, no studies are known to us that have investigated the effect of previous pathogen infestation (and thus likely induction of SA) on the ability of plants to induce volatiles that attract carnivores.

There is no evidence for an interaction of ethylene with JA-mediated volatile induction so far. In tobacco, ethylene treatment did not influence the MeJA-induced emission of (-)-cis-α-bergamotene. This contrasts to the interference of ethylene with MeJA induced production of nicotine (Kahl et al., 2000).

5. Interaction between direct and indirect defence

Plants are attacked by a wide range herbivores and have evolved a variety of defences. The coordination of these defences seems to be a formidable task and may have several conflicts. The tailoring of defences seems to be a complex optimisation problem, the outcome of which is dependent on the intensity and frequency of different types of attackers and their natural enemies.

For instance, plants are attacked by specialist and generalist herbivores. Specialist herbivores are usually well adapted to the defences of their host plant. They may even exploit secondary compounds that provide protection against generalist herbivores as token stimuli to recognise their host plant. Oviposition in Pieris butterflies is stimulated by glucosinolates of their cruciferous host plants and for caterpillars these compounds act as feeding stimulants (Chew and Renwick, 1995). Specialist herbivores can also exploit secondary metabolites of their host plant in their own defence. Manduca sexta caterpillars derive protection from pathogens and parasitoids by the intake of nicotine from tobacco leaves (Barbosa et al., 1986; Krischik et al., 1988). In addition, direct defences may interfere with natural enemies of herbivores without an involvement of the herbivore. Glandular trichomes on tomato stems are a defence against herbivores, but in addition they kill the majority of predatory mites that forage on the plant by entrapment. Therefore, this direct defence is incompatible with indirect defence through predatory mites. This shows that direct defences can interfere with the effectiveness of indirect defences and one may wonder whether there is a negative correlation between investments in direct and indirect defence. In this context it is interesting to note that tomato and cabbage, that have a strong direct defence, do not emit novel volatiles in response to herbivory compared to mechanical damage. As a consequence the information content is much lower than for plants like Lima bean or maize that emit novel compounds that dominate the blend in response to herbivory (Dicke and Vet, 1999). At the individual plant level, an uncoupling of direct and indirect defence appears to be possible. In tobacco plants, damage by M. sexta caterpillars or treatment with their regurgitant results in an attenuation of induced direct defence (nicotine production) that is mediated by herbivore-induced ethylene. In contrast, there is no such effect on the induction of indirect defence (Kahl et al., 2000).

6. Interaction between defences against pathogens and herbivores

Plants are also attacked by a wide range of pathogens. The defences induced against pathogens and herbivores may act synergistically (e.g. Karban *et al*., 1987) or antagonistically (e.g. Thaler *et al*., 1999). Yet, both in direct and indirect defences against herbivores and in defences against pathogens similar combinations of signal-transduction pathways may be induced. These pathways may act antagonistically, such as in the interference between SA- and JA-mediated defences. The current knowledge suggests that at certain points in the signal-transduction pathways, switch points are present that influence the final outcome of the collective signal transduction pathways. With an increasing knowledge of signal transduction in induced defences against pathogens as well as those against herbivores, it will be important to unravel where these switch points are, where cross-talk between pathways occurs and especially how cross-talk is regulated. This will be a major challenge for the forthcoming years. It will be important to investigate different plant species and populations, especially when different populations are under different relative pressures of herbivores and pathogens.

7. Comparative analysis of signal transduction in induction of direct and indirect defence: model systems

The signal transduction pathways involved in induction of direct defence have been well studied and there is a fertile area for the study of cross-talk. The research on signal-transduction in induced indirect defence is rapidly advancing and bridging the gap with the knowledge in direct defences. However, the two research fields have developed independently, mostly with different model systems. In induced direct defence the best studied model plants are the solanaceous plants tomato and tobacco, while in induced indirect defence these comprise e.g. Lima bean, maize, cotton and cabbage. There seems to be a difference in the way solanaceous and cruciferous plants employ indirect defence (few novel compounds induced) versus that of plants such as Lima bean and maize (major novel compounds induced by herbivory). To allow a better comparison of signal transduction in induced direct and indirect defence, common model systems are highly needed. Several model systems seem to be good candidates.

Tobacco has been well studied for induced direct defence against pathogens and insects and the signal transduction pathways involved (reviewed by Karban and Baldwin, 1997; Baldwin, 1999; Baldwin and Preston, 1999). Recently, these studies have also incorporated the induction of indirect defence (Kahl et al., 2000). This will make tobacco a very interesting model system for a comparative analysis.

There is abundant knowledge on the induction of direct defences against pathogens and herbivores in tomato (Pearce et al., 1991; Ryan, 1992; Jones et al., 1994; Stout et al., 1999; Thaler et al., 1999) and several mutants and transgenes are available (Orozco-Cardenas et al., 1993; McGurl et al., 1994; Howe et al., 1996). In addition, studies on the induction of indirect defence are emerging (Dicke et al., 1998; Thaler, 1999; Thaler et al., 1999). Therefore, tomato will be an interesting model system too.

Arabidopsis thaliana has been well studied for direct defence against pathogens (Dangl, 1993). Studies on direct defence against insects are emerging as well (Grant-Petersson and Renwick, 1996; Mauricio and Rausher, 1997). In addition, there is abundant knowledge on direct defence in other crucifers (e.g. Shapiro and DeVay, 1987; Bodnaryk, 1992; Palaniswamy and Lamb, 1993; Avdiushko et al., 1997; Agrawal, 1998) against herbivores and pathogens. Recently, the first demonstration of induced indirect defence has been made in Arabidopsis (Van Loon et al., 2000a; and see Chapter 3). Together with the presence of various mutants and transgenes that are affected in signal transduction pathways and the near completion of the genomic analysis, Arabidopsis will become an important model plant for comparative studies on signal transduction in direct and indirect defence.

When concentrating on such model plants, it remains important to consider other plant species as well. The above-mentioned three plant species all have in common that they have a well-developed direct defence. The blend of volatiles induced in tomato plants by spider mites is dominated by non-novel compounds (Dicke et al., 1998) and thus contrasts to the situation in plants like Lima bean, cucumber and maize (Dicke et al., 1990b; Turlings et al., 1990; Dicke et al., 1999). Therefore, it remains important to include the latter three plant species as well to enable a comparison among plants with different types of induced indirect defence.

8. Major questions to be addressed

Developments in the research on signal transduction in induced defence against herbivorous insects have been numerous in the past decade. Induction of direct and indirect defence appear to share many signal transduction pathways. The octadecanoid signal-transduction pathway seems to be the major pathway involved in induced defences against insects. However, there are clear indications that other pathways play an essential role as well. Recent work demonstrates that the most important signalling molecule in addition to JA is SA. This is clear from e.g. the increasing number of reports on the emission of MeSA from herbivore-damaged plants. The formation of MeSA in response to herbivory as well as the influence on JA-mediated signalling deserves further investigation.

The induction of indirect defence against herbivorous insects seems to be more specific than the induction of direct defence. In many cases there is a clearly differential response to mechanical damage versus the response to herbivory or herbivore elicitors. The compositions of volatile blends can vary substantially with the species or instar of herbivore that damages the plant and this variation can have considerable consequences for carnivore attraction and thus for defence effectiveness. What signal transduction events mediate these subtly differential responses of plants will be a rewarding subject. Most likely the involvement of signal molecules from different signal transduction pathways will be found.

A signal molecule that has not received much attention to date, is ethylene. This molecule is involved in the induction of defences against pathogens or in responses to non-pathogenic microorganisms (Pieterse *et al.*, 1998) and modulates the induction of nicotine in tobacco plants (Kahl *et al.*, 2000).

The rapid development in research on signal transduction in induced defence against insects enables the integration with research on induced defences against pathogens. This will be very important, because in nature plants are exposed to a gamut of attackers that include pathogens and herbivores. Investigating how plants integrate defences against all these attackers will be an important step.

In indirect defence, extensive knowledge is present on the attraction of carnivores to complete blends of volatiles emitted by herbivore-damaged or elicitor treated plants. However, knowledge on the bio-active compounds within the blend is limited to a few studies (Dicke and Van Loon, 2000). The use of elicitors that selectively induce certain blend components as well as mutants or transgenes that are modified in signal-transduction pathways or in biosynthetic pathways (Chapter 4, 5 and 6) will provide excellent opportunities to elucidate which compounds are most important in attracting carnivores and how variation affects this.

With significant progress in the knowledge of mechanisms of induced plant defences, e.g., in molecular genetics, biochemistry, plant physiology, many tools will become available to investigate the function of these plant traits. Together with progress in the options of manipulating plant genotype, the ultimate step may be made, i.e. investigating the effects on plant phenotype in its interactions with biotic and abiotic components of the environment.

References

Agelopoulos, N. G. and Keller, M. A. (1994). Plant-natural enemy association in the tritrophic system, *Cotesia rubecula-Pieris rapae*-Brassicaceae (Crucifera): III. Collection and identification of plant and frass volatiles. *J. Chem. Ecol.* 20: 1955-1967.

Agrawal, A. A. (1998). Induced responses to herbivory and increased plant performance. *Science* 279: 1201-1202.

Agrawal, A.A., Tuzun, S. and Bent, E. (1999). *Induced Plant Defenses Against Pathogens and Herbivores*. APS Press,St. Paul MI.

Alborn, T., Turlings, T. C. J., Jones, T. H., Steinhagen, G., Loughrin, J. H. and Tumlinson, J. H. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276: 945-949.

Arimura, G., Ozawa, R., Shimoda, T., Nishioka, T., Boland, W. and Takabayashi, J. (2000). Herbivory-induced volatiles elicit defence genes in lima bean leaves. *Nature* 406: 512-515.

Avdiushko, S., Croft, K. P. C., Brown, G. C., Jackson, D. M., Hamiltonkemp, T. R. and Hildebrand, D. (1995). Effect of volatile methyl jasmonate on the oxylipin pathway in tobacco, cucumber, and arabidopsis. *Plant Physiol.* 109: 1227-1230.

Avdiushko, S. A., Brown, G. C., Dahlman, D. L. and Hildebrand, D. F. (1997). Methyl jasmonate exposure induces insect resistance in cabbage and tobacco. *Environ. Entomol.* 26: 642-654.

Baldwin, I. T. (1990). Herbivory simulations in ecological research. *Trends Ecol. Evol.* 5: 91-93.

Baldwin, I.T. (1999). Inducible nicotine production in native *Nicotiana* as an example of adaptive phenotypic plasticity. *J. Chem. Ecol.* 25: 3-30.

Baldwin, I. T. and Preston, C. A. (1999). The eco-physiological complexity of plant responses to insect herbivores. *Planta* 208: 137-145.

Baldwin, I. T., Zhang, Z. P., Diab, N., Ohnmeiss, T. E., McCloud, E. S. , Lynds, G. Y. and Schmelz, E. A. (1997). Quantification, correlations and manipulations of wound-induced changes in jasmonic acid and nicotine in *Nicotiana sylvestris*. *Planta* 201: 397-404.

Barbosa, P., Saunders, J. A., Kemper, J., Trumbule, R., Olechno, J. and Martinat, P. (1986). Plant allelochemicals and insect parasitoids. Effects of nicotine on *Cotesia congregata* (Say) (Hymenoptera: Braconidae) and *Hyposoter annulipes* (Cresson) (Hymenoptera: Ichneumonidae). *J. Chem. Ecol.* 12: 1319-1328.

Barry, C.S., Blume, B., Bouzayen, M., Cooper, W., Hamilton, A.J. and Grierson, D. (1996). Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. *Plant J.* 9: 525 - 535.

Bell, E., Creelman, R.A. and Mullet, J.E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 92: 8675-8679.

Berenbaum, M.R. and Zangerl, A.R. (1999). Coping with life as a menu option: inducible defences of the wild parsnip. In *The Ecology and Evolution of Inducible Defenses*, eds. R. Tollrian and C.D. Harvell, pp. 10-32. Princeton University Press, Princeton, NJ.

Berger, S., Bell, E. and Mullet, J. E. (1996). Two methyl jasmonate-insensitive mutants show altered expression of AtVsp in response to methyl jasmonate and wounding. *Plant Physiol* 111: 525-531.

Bergey, D.R., Howe, G.A. and Ryan, C.A. (1996). Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. *Proc. Natl. Acad. Sci. USA* 93: 12053-12058.

Bi, J. L., Murphy, J. B. and Felton, G. W. (1997). Does salicylic acid act as a signal for induced resistance in cotton to *Helicoverpa zea*? *J. Chem. Ecol.* 23: 1805-1818.

Birkenmeier, G. F. and Ryan, C. A. (1998). Wound signaling in tomato plants - Evidence that ABA is not a primary signal for defense gene activation. *Plant Physiol*. 117: 687-693.

Blaakmeer, A., Geervliet, J. B. F., van Loon, J. J. A., Posthumus, M. A. , van Beek, T. A. and de Groot, A. E. (1994). Comparative headspace analysis of cabbage plants damaged by two species of *Pieris* caterpillars: consequences for in-flight host location by *Cotesia* parasitoids. *Entomol. Exp. Appl.* 73: 175-182.

Blechert, S., Brodschelm, W., Holder, S., Kammerer, L., Kutchan, T. M., Mueller, M. J., Xia, Z. Q. and Zenk, M. H. (1995). The octadecanoic pathway: signal molecules for the regulation of secondary pathways. *Proc. Natl. Acad. Sci. USA* 92: 4099-4105.

Bodnaryk, R. P. (1992). Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry* 31: 2671-2677.

Boland, W., Feng, Z., Donath, J. and Gäbler, A. (1992). Are acyclic C11 and C16 homoterpenes plant volatiles indicating herbivory? *Naturwissenschaften* 79: 368-371.

Boland, W., Hopke, J., Donath, J., Nueske, J. and Bublitz, F. (1995). Jasmonic acid and coronatin induce odor production in plants. *Angew Chem.* 34: 1600-1602.

Boland, W., Koch, T., Krumm, T., Piel, J. and Jux, A. (1999). Induced biosynthesis of insect semiochemicals in plants. In *Insect-Plant Interactions and Induced Plant Defence* (Novartis Foundation Symposium 223),, eds. D.J. Chadwick and J. Goode, pp. 110-126. Wiley, Chicester.

Bolter, C. J., Dicke, M., van Loon, J.J. A., Visser, J. H. and Posthumus, M. A. (1997). Attraction of Colorado potato beetle to herbivore damaged plants during herbivory and after its termination. *J. Chem. Ecol.* 23: 1003-1023.

Bouwmeester, H.J., Verstappen, F., Posthumus, M.A. and Dicke, M. (1999). Spider-mite induced (3S)-(*E*)-nerolidol synthase activity in cucumber and Lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. *Plant Physiol.* 121: 173-180.

Broadway, R. M., Duffey, S. S., Pearce, G. and Ryan, C. A. (1986). Plant proteinase inhibitors: a defense against herbivorous insects? *Entomol. exp. appl.* 41: 33-38.

Bruin, J., Sabelis, M. W. and Dicke, M. (1995). Do plants tap SOS signals from their infested neighbours? *Trends Ecol. Evol.* 10: 167-170.

Chao, W.S., Gu, Y.-Q., Pautot, V., Bray, E.A. and Walling, L.L. (1999). Leucine aminopeptidase RMAs, proteins and activities increase in response to water deficit, salinity and the wound signals systemin, methyl jasmonate and abscisic acid. *Plant Physiol.* 120: 979-992.

Chew, F.S. and Renwick, J. A. A. (1995). Host plant choice in Pieris butterflies214-238. Chapman and Hall, New York.

Constabel, C. P., Bergey, D. R. and Ryan, C. A. (1995). Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. *Proc. Natl. Acad. Sci. USA* 92: 407-411.

Constabel, C.P., Yip, L. and Ryan, C.A. (1998). Prosystemin from potato, black nightshade, and bell pepper: primary structure and biological activity of predicted systemin polypeptides. *Plant Mol. Biol.* 36: 55-62.

Creelman, R. A., Tierney, M. L. and Mullet, J. E. (1992). Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. *Proc. Natl. Acad. Sci. USA* 89: 4938-4941.

Croft, K. P., Juttner, F. and Slusarenko, A. J. (1993). Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv. phaseolicola. *Plant Physiol.* 101: 13-24.

Dangl, J. L. (1993). The emergence of Arabidopsis thaliana as a model for plant-pathogen interactions. *Adv.Plant Pathol.* 10: 127-156.

De Moraes, C. M., Lewis, W. J., Paré, P. W., Alborn, H. T. and Tumlinson, J. H. (1998). Herbivore-infested plants selectively attract parasitoids. *Nature* 393: 570-573.

Degenhardt, J. and Gershenzon, J. (2000). Demonstration and characterization of (*E*)-nerolidol synthase from maize: a herbivore-inducible terpene synthase participating in (3*E*)-4,8-dimethyl-1,3,7-nonatriene biosynthesis. *Planta* 210: 815-822.

Dicke, M. (1986). Volatile spider-mite pheromone and host-plant kairomone, involved in spaced-out gregariousness in the spider mite *Tetranychus urticae*. *Physiol. Entomol.* 11: 251-262.

Dicke, M. (1994). Local and systemic production of volatile herbivore-induced terpenoids: Their role in plant-carnivore mutualism. *J. Plant Physiol.* 143: 465-472.

Dicke, M. (1999a). Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? *Entomol. Exp. Appl.* 92: 131-142.

Dicke, M. (1999b). Direct and indirect effects of plants on performance of beneficial organisms. In *Handbook of Pest Management*, eds. J.R. Ruberson, pp. 105-153. Marcel Dekker, New York.

Dicke, M. (1999c). Evolution of induced indirect defence of plants. In *The Ecology and Evolution of Inducible Defenses*, eds. R. Tollrian and C.D. Harvell, pp. 62-88. Princeton University Press, Princeton, NJ.

Dicke, M. and Sabelis, M. W. (1988). How plants obtain predatory mites as bodyguards. *Neth. J. Zool.* 38: 148-165.

Dicke, M. and Vet, L.E.M. (1999). Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore. In *Herbivores: Between Plants and Predators*, eds. H. Olff, V.K. Brown and R.H. Drent, pp. 483-520. Blackwell Science, Oxford, UK.

Dicke, M. and Van Loon, J.J.A. (2000). Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. *Entomol. Exp. Appl.* 97: 237-249.

Dicke, M., Sabelis, M. W., Takabayashi, J., Bruin, J. and Posthumus, M. A. (1990a). Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control. *J. Chem. Ecol.* 16: 3091-3118.

Dicke, M., van Beek, T. A., Posthumus, M. A., Ben Dom, N., van Bokhoven, H. and de Groot, A. E. (1990b). Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16: 381-396.

Dicke, M., Baarlen, P. van, Wessels, R. and Dijkman, H. (1993). Herbivory induces systemic production of plant volatiles that attract predators of the herbivore: extraction of endogenous elicitor. *J. Chem. Ecol.* 19: 581-599.

Dicke, M., Takabayashi, J., Posthumus, M.A., Schütte, C. and Krips, O.E. (1998). Plant-phytoseiid interactions mediated by prey-induced plant volatiles: variation in production of cues and variation in responses of predatory mites. *Exp. Appl. Acarol.* 22: 311-333.

Dicke, M., Gols, R., Ludeking, D. and Posthumus, M. A. (1999). Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25: 1907-1922.

Doares, S. H., Narvaez-Vasquez, J., Conconi, A. and Ryan, C. A. (1995). Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant Physiol.* 108: 1741-1746.

Donath, J. and Boland, W. (1994). Biosynthesis of acyclic homoterpenes in higher plants parallels steroid hormone metabolism. *J. Plant Physiol.* 143: 473-478.

Du, Y., Poppy, G. M., Powell, W., Pickett, J. A., Wadhams, L. J. and Woodcock, C. M. (1998). Identification of semiochemicals released during aphid feeding that attract parasitoid Aphidius ervi. J. Chem. Ecol. 24: 1355-1368.

Ecker, J. R. and Davis, R. W. (1987). Plant defense genes are regulated by ethylene. *Proc. Natl. Acad. Sci. USA* 84: 5202-5206.

Eichenseer, H., Mathews, M.C., Bi, J.L., Murphy, B. and Felton, G.W. (1999). Salivary glucose oxidase: multifunctional roles for *Helicoverpa zea*? *Arch. Insect Biochem. Physiol.* 42: 99-109.

Engelberth, J., Koch, T., Kühnemann, F. and Boland, W. (2000). Channel-forming peptaibols are a novel class of potent elicitors of plant secondary metabolism and tendril coiling. *Angew. Chem. Intl. Ed.* 39: 1860-1862.

Enyedi, A. J., Yalpani, N., Silverman, P. and I., Raskin. (1992). Signal molecules in systemic plant resistance to pathogens and pests. *Cell* 70: 879-886.

Farmer, E. E. and Ryan, C. A. (1990). Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc. Natl. Acad. Sci. USA* 87: 7713-7716.

Farmer, E. E. and Ryan, C. A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* 4: 129-134.

Felix, G. and Boller, T. (1995). Systemin induces rapid ion fluxes and ethylene biosynthesis in *Lycopersicon peruvianum* cells. *Plant J.* 7: 381-389.

Felton, G.W. and Eichenseer, H. (1999). Herbivore saliva and its effect on plant defense against herbivores and pathogens. In *Induced plant defenses against pathogens and herbivores. Biochemistry, ecology and agriculture*, eds. A.A. Agrawal, S. Tuzun and E. Bent, pp. 19-36. APS Press, St. Paul, MN.

Geervliet, J. B. F., Vet, L. E. M. and Dicke, M. (1994). Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomol. Exp. Appl.* 73: 289-297.

Geervliet, J.B.F., Posthumus, M.A., Vet, L.E.M. and Dicke, M. (1997). Comparative analysis of headspace volatiles from different caterpillar-infested and uninfested food plants of *Pieris* species. *J. Chem. Ecol.* 23: 2935-2954.

Gershenzon, J. and Croteau, R. (1991). Terpenoids. In *Herbivores: their interactions with secondary plant metabolites*, vol. 1, eds. G.A. Rosenthal and M.R. Berenbaum, pp. 165-219. Acad. Press, New York.

Gols, R., Posthumus, M.A. and Dicke, M. (1999). Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent *Phytoseiulus persimilis. Entomol. Exp. Appl.* 93: 77-86.

Grant-Petersson, J. and Renwick, J. A. A. (1996). Effects of ultraviolet-b exposure of *Arabidopsis thaliana* on herbivory by two crucifer-feeding insects (Lepidoptera). *Environ. Entomol.* 25: 135-142.

Green, T. R. and Ryan, C. A. (1971). Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science* 175: 776-777.

Grostal, P. and O'Dowd, D. J. (1994). Plants, mites and mutualism: leaf domatia and the abundance and reproduction of mites on *Viburnum tinus* (Caprifoliaceae). *Oecologia* 97: 308-315.

Gundlach, H., Muller, M. J., Kutchan, T. M. and Zenk, M. H. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc. Natl. Acad. Sci. USA* 89: 2389-2393.

Hartley, S. E. and Lawton, J. H. (1991). Biochemical aspects and significance of the rapidly induced accumulation of phenolics in birch foliage. In *Phytochemical induction by herbivores*, eds. D.W. Tallamy and M.J. Raupp, pp. 105-132. John Wiley and Sons, New York.

Harvey, J.A. (2000). Dynamic effects of parasitism by an endoparasitoid wasp on the development of two host species: implications for host quality and parasitoid fitness. *Ecol. Entomol.* 25: 267-278.

Hatanaka, A., Kajiwara, T. and Sekiya, J. (1987). Biosynthetic pathway for C6-aldehydes formation from linolenic acid in green leaves. *Chem. Phys. Liphids* 44: 341-361.

Hibi, N., Higashiguchi, S., Hashimoto, T. and Yamada, Y. (1994). Gene expression in tobacco low-nicotine mutants. *Plant Cell* 6: 723 - 735.

Hildebrand, D. F., Brown, G. C., Jackson, D. M. and Hamilton-Kemp, T. R. (1993). Effects of some leaf-emitted volatile compounds on aphid population increase. *J. Chem. Ecol.* 19: 1875-1887.

Hölldobler, B. and Wilson, E. O. (1990). *The Ants*. Harvard University Press,Cambridge MA.

Hopke, J., Donath, J., Blechert, S. and Boland, W. (1994). Herbivore-induced volatiles: the emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by a ß-glucosidase and jasmonic acid. *FEBS Letters* 352: 146-150.

Howe, G. A., Lightner, J., Browse, J. and Ryan, C. A. (1996). An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. *Plant Cell* 8: 2067-2077.

Janssen, A. (1999). Plants with spider-mite prey attract more predatory mites than clean plants under greenhouse conditions. *Entomol. Exp. Appl.* 90: 191-198.

Jones, D. A., Thomas, C. M., Hammond-Kosack, K. E., Balint-Kurti, P. J. and Jones, J. D. G. (1994). Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* 266: 789-793.

Jongsma, M.A., Bakker, P.L., Visser, B. and Stiekema, W.J. (1994). Trypsin inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus infection. *Planta* 195: 29-35.

Jongsma, M. A., Bakker, P.L., Peters, J., Bosch, D. and Stiekema, W. J. (1995). Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. *Proc. Natl. Acad. Sci. USA* 92: 8041-8045.

Kahl, J., Siemens, D.H., Aerts, R.J., Gäbler, R., Kühnemann, F., Preston, C.A. and Baldwin, I.T. (2000). Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. *Planta* 210: 336-342.

Kaiser, R. A. J. (1993). On the scent of orchids. In *Bioactive volatile compounds from plants*, eds. R. Teranishi, R.G. Buttery and H. Sugisawa, pp. ACS Symposium Series 525: 240-268. Am. Chem. Soc., Washington, D.C.

Karban, R. and Baldwin, I.T. (1997). *Induced responses to herbivory.* Chicago University Press,Chicago.

Karban, R., Adamchak, R. and Schnathorst, W. C. (1987). Induced resistance and interspecific competition between spider mites and a vascular wilt fungus. *Science* 235: 678-680.

Karban, R., Baldwin, I.T., Baxter, K.J., Laue, G. and Felton, G.W. (2000). Communication between plants: Induced resistance in wild tobacco plants following clipping of neighboring sagebrush. *Oecologia* 125: 66-71.

Koptur, S. (1992). Extrafloral nectary-mediated interactions between insects and plants. In *Insect-Plant Interactions*, vol. 4, eds. E.A. Bernays, pp. 81-129. CRC Press, Boca Raton, Florida.

Korth, K. L. and Dixon, R. A. (1997). Evidence for chewing insect-specific molecular events distinct from a general wound response in leaves. *Plant Physiol*. 115: 1299-1305.

Krips, O.E., Willems, P.E.L., Gols, R., Posthumus, M.A. and Dicke, M. (1999). The response of *Phytoseiulus persimilis* to spider-mite induced volatiles from gerbera: influence of starvation and experience. *J. Chem. Ecol.* 25: 2623-2641.

Krischik, V. A., Barbosa, P. and Reichelderfer, C. F. (1988). Three trophic level interactions: allelochemicals, *Manduca sexta* (L.), and *Bacillus thuringiensis* var. kurstaki Berliner. *Environ. Entomol.* 17: 476-482.

Laudert, D. and Weiler, E.W. (1998). Allene oxide synthase: a major control point in *Arabdopsis thaliana* octadecanoid signaling. *Plant J.* 15: 675-684.

Lichtenthaler, H.K. (1999). The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50: 47-65.

Lin, H., Kogan, M. and Fischer, D. (1990). Induced resistance in soybean to the Mexican bean beetle (Coleptera: Coccinellidae): comparison of inducing factors. *Environ. Entomol.* 19: 1852-1857.

Loader, C. and Damman, H. (1991). Nitrogen content of food plants and vulnerability of *Pieris rapae* to natural enemies. *Ecology* 72: 1586-1590.

Martin, W. R., Morgan, P. W., Sterling, W. L. and Meola, R. W. (1988). Stimulation of ethylene production in cotton by salivary enzymes of the cotton fleahopper (Heteroptera: Miridae). *Environ. Entomol.* 17: 930-935.

Mattiacci, L., Dicke, M. and Posthumus, M. A. (1994). Induction of parasitoid attracting synomone in brussels sprouts plants by feeding of *Pieris brassicae* larvae: role of mechanical damage and herbivore elicitor. *J. Chem. Ecol.* 20: 2229-2247.

Mattiacci, L., Dicke, M. and Posthumus, M. A. (1995). beta-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proc. Natl. Acad. Sci. USA* 92: 2036-2040.

Mauricio, R. and Rausher, M. D. (1997). Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution* 5: 1435-1444.

McCall, P. J., Turlings, T. C. J., Lewis, W. J. and Tumlinson, J. H. (1993). Role of plant volatiles in host location by the specialist parasitoid *Microplitis croceipes* Cresson (Braconidae: Hymenoptera). *J. Insect Beh.* 6: 625-639.

McCall, P. J., Turlings, T. C. J., Loughrin, J., Proveaux, A. T. and Tumlinson, J. H. (1994). Herbivore-induced volatile emissions from cotton (*Gossypium hirsutum* L.) seedlings. *J. Chem. Ecol.* 20: 3039-3050.

McCloud, E. S. and Baldwin, I. T. (1997). Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*. *Planta* 203: 430-435.

McConn, M., Creelman, R. A., Bell, E., Mullet, J. E. and Browse, J. (1997). Jasmonate is essential for insect defense in *Arabidopsis*. *Proc. Natl. Acad. Sci.* USA 94: 5473-5477.

McGurl, B., Pearce, G., Orozco-Cardenas, M. and Ryan, C. A. (1992). Structure, expression, and antisense inhibition of the systemin precursor gene. *Science* 255: 1570-1573.

McGurl, B., Orozco-Cardenas, M., Pearce, G. and Ryan, C. A. (1994). Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. *Proc. Natl. Acad. Sci. USA* 91: 9799-9802.

Meiners, T. and Hilker, M. (2000). Induction of plant synomones by oviposition of a phytophagous insect. *J. Chem. Ecol.* 26: 221-232.

Mueller, M. J. (1997). Enzymes involved in jasmonic acid biosynthesis. *Physiol. Plant.* 100: 653-663.

Narvaez-Vasquez, J., Pearce, G., Orozco-Cardenas, M. L., Franceschi, V. R. and Ryan, C. A. (1995). Autoradiographic and biochemical evidence for the systemic translocation of systemin in tomato plants. *Planta* 195: 593-600.

O'Donnell, P. J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H. M. O. and Bowles, D. J. (1996). Ethylene as a signal mediating the wound response of tomato plants. *Science* 274: 1914-1917.

Orozco-Cardenas, M., McGurl, B. and Ryan, C. A. (1993). Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. *Proc. Natl. Acad. Sci. USA* 90: 8273-8276.

Ozawa, R., Arimura, G., Takabayashi, J., Shimoda, T. and Nishioka, T. (2000). Involvement of jasmonate- and salicylate-related signaling pathway for the production of specific herbivore-induced volatiles in plants. *Plant Cell Physiol.* 41: 391-398.

Palaniswamy, P. and Lamb, R. J. (1993). Wound-induced antixenotic resistance to flea beetles, *Phyllotreta cruciferae* (Goeze) (Coleoptera: Chrysomelidae) in crucifers. *Can. Entomol.* 125: 903-912.

Paré, P. W. and Tumlinson, J. H. (1997). De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol.* 114: 1161-1167.

Pearce, G., Strydom, D., Johnson, S. and Ryan, C. A. (1991). A Polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* 253: 895-898.

Peña-Cortés, H., Albrecht, T., Prat, S., Weiler, E. W. and Willmitzer, L. (1993). Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. *Planta* 191: 123-128.

Peña-Cortés, H., Sanchez-Serrano, J. J., Mertens, R., Willmitzer, L. and Prat, S. (1989). Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. *Proc. Natl. Acad. Sci. USA* 86: 9851-9855.

Piel, J., Atzorn, R., Gabler, R., Kuhnemann, F. and Boland, W. (1997). Cellulysin from the plant parasitic fungus *Trichoderma viride* elicits volatile biosynthesis in higher plants via the octadecanoid signalling cascade. *FEBS Letters* 416: 143-148.

Pieterse, C. M. J., van Wees, S. C. M., van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P. J. and van Loon, L. C. (1998). A novel signaling pathway controlling induced systemic resistance in arabidopsis. *Plant Cell* 10: 1571-1580.

Rieske, L. K. and Raffa, K. F. (1995). Ethylene emission by a deciduous tree, *Tilia americana*, in response to feeding by introduced basswood thrips, *Thrips calcaratus. J. Chem. Ecol*. 21: 187-197.

Rojo, E., Leon, J. and Sanchez-Serrano, J.J. (1999). Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana. Plant J.* 20: 135-142.

Röse, U. S. R., Manukian, A., Heath, R. R. and Tumlinson, J. H. (1996). Volatile semiochemicals released from undamaged cotton leaves - a systemic response of living plants to caterpillar damage. *Plant Physiol*. 111: 487-495.

Ryan, C.A. (1974). Assay and biochemical properties of the proteinase inhibitor-inducing factor, a wound hormone. *Plant Physiol.* 54: 328-332.

Ryan, C. A. (1990). Protease inhibitors in plants: genes for improving defenses against insect and pathogens. *Annu. Rev. Phytopathol.* 28: 425-429.

Ryan, C. A. (1992). The search for the proteinase inhibitor-inducing factor, PIIF. *Plant Mol. Biol.* 19: 123-133.

Sabelis, M. W. and Van de Baan, H. E. (1983). Location of distant spider mite colonies by phytoseiid predators: demonstration of specific kairomones emitted by *Tetranychus urticae* and *Panonychus ulmi*. *Entomol. Exp. Appl.* 33: 303-314.

Sabelis, M. W. and Dicke, M. (1985). Long-range dispersal and searching behaviour. In *Spider Mites: Their Biology, Natural Enemies and Control*. World Crop Pests 1A, eds. Helle W. and M.W. Sabelis, pp. 141-160. Elsevier, Amsterdam.

Sabelis, M. W. and Afman, B. P. (1994). Synomone-induced suppression of take-off in the phytoseiid mite *Phytoseiulus persimilis* Athias-Henriot. *Exp. Appl. Acarol.* 18: 711-721.

Sabelis, M. W., Afman, B. P. and Slim, P. J. (1984a). Location of distant spider mite colonies by *Phytoseiulus persimilis*: localization and extraction of a kairomone. *Acarology* VI 1: 431-440.

Sabelis, M. W., Vermaat, J. E. and Groeneveld, A. (1984b). Arrestment responses of the predatory mite, *Phytoseiulus persimilis*, to steep odour gradients of a kairomone. *Physiol. Entomol.* 9: 437-446.

Sabelis, M.W., van Baalen, M., Bakker, F.M., Bruin, J., Drukker, B., Egas, M., Janssen, A.R.M., Lesna, I.K., Pels, B., Van Rijn, P. and Scutareanu, P. (1999). The evolution of direct and indirect plant defence against herbivorous arthropods. In *Herbivores: Between plants and predators*, eds. H. Olff, V.K. Brown and R.H. Drent, pp. 109-166. Blackwell Science, Oxford.

Sano, H. and Ohashi, Y. (1995). Involvement of small GTP-binding proteins in defense signal-transduction pathways of higher plants. *Proc. Natl. Acad. Sci. USA* 92: 4138 - 4144.

Schoonhoven, L.M., Jermy, T. and Van Loon, J.J.A. (1998). *Insect-Plant Biology. From Physiology to Evolution*. Chapman and Hall,London.

Scutareanu, P., Drukker, B., Bruin, J., Posthumus, M. A. and Sabelis, M. W. (1997). Volatiles from *Psylla*-infested pear trees and their possible involvement in attraction of anthocorid predators. *J. Chem. Ecol.* 23: 2241-2260.

Sembdner, G. and Parthier, B. (1993). The biochemistry and the physiological and molecular actions of jasmonates. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44: 569-589.

Shapiro, A. M. and DeVay, J. E. (1987). Hypersensitivity reaction of *Brassica nigra* L. (Cruciferae) kills eggs of *Pieris* butterflies (Lepidoptera: Pieridae). *Oecologia* 71: 631-632.

Shiojiri, K., Takabayashi, J., Yano, S. and Takafuji, A. (2000). Flight response of parasitoids toward plant-herbivore complexes: A comparative study of two parasitoid-herbivore systems on cabbage plants. *Appl. Entomol. Zool.* 35: 87-92.

Shonle, I. and Bergelson, J. (1995). Interplant communication revisited. *Ecology* 76: 2660-2663.

Shulaev, V., Silverman, P. and Raskin, I. (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* 385: 718-721.

Steinberg, S., Dicke, M. and Vet, L. E. M. (1993). Relative importance of infochemicals from first and second trophic level in long-range host location by the larval parasitoid *Cotesia glomerata*. *J. Chem. Ecol.* 19: 47-59.

Stout, M. J., Workman, J. and Duffey, S. S. (1994). Differential induction of tomato foliar proteins by arthropod herbivores. *J. Chem. Ecol.* 20: 2575-2594.

Stout, M. J., Fidantsef, A. L., Duffey, S. S. and Bostock, R. M. (1999). Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiol. Mol. Plant Pathol.* 54: 115-130.

Takabayashi, J. and Dicke, M. (1996). Plant-carnivore mutualism through herbivore-induced carnivore attractants. *Trends Plant Sci.* 1: 109-113.

Takabayashi, J., Dicke, M. and Posthumus, M. A. (1991). Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: relative influence of plant and herbivore. *Chemoecology* 2: 1-6.

Takabayashi, J., Dicke, M. and Posthumus, M. A. (1994a). Volatile herbivore-induced terpenoids in plant-mite interactions: Variation caused by biotic and abiotic factors. *J. Chem. Ecol.* 20: 1329-1354.

Takabayashi, J., Dicke, M., Takahashi, S., Posthumus, M. A. and van Beek, T. A. (1994b). Leaf age affects composition of herbivore-induced synomones and attraction of predatory mites. *J. Chem. Ecol.* 20: 373-386.

Takabayashi, J., Takahashi, S., Dicke, M. and Posthumus, M. A. (1995). Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. *J. Chem. Ecol.* 21: 273-287.

Thaler, J. S. (1999). Jasmonate-inducible plant defenses cause increased parasitism of herbivores. *Nature* 399: 686-688.

Thaler, J. S., Fidantsef, A. L., Duffey, S. S. and Bostock, R. M. (1999). Trade-offs in plant defense against pathogens and herbivores: A field demonstration of chemical elicitors of induced resistance. *J. Chem. Ecol.* 25: 1597-1609.

Turlings, T. C. J. and Tumlinson, J. H. (1992). Systemic release of chemical signals by herbivore-injured corn. *Proc. Natl. Acad. Sci. USA* 89: 8399-8402.

Turlings, T. C. J. and Benrey, B. (1998). Effects of plant metabolites on behavior and development of parasitic wasps. *Ecoscience* 5: 321-333.

Turlings, T. C. J., Tumlinson, J. H. and Lewis, W. J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250: 1251-1253.

Turlings, T. C. J., Tumlinson, J. H., Eller, F. J. and Lewis, W. J. (1991). Larval-damaged plants: source of volatile synomones that guide the parasitoid *Cotesia marginiventris* to the micro-habitat of its hosts. *Entomol. Exp. Appl.* 58: 75-82.

Turlings, T. C. J., Wäckers, F. L., Vet, L. E. M., Lewis, W. J. and Tumlinson, J. H. (1993). Learning of host-finding cues by hymenopterous parasitoids. In *Insect learning: ecological and evolutionary perspectives*, eds. D.R. Papaj and A.C. Lewis, pp. 51-78. Chapman and Hall, New York.

Turlings, T. C. J., Loughrin, J. H., McCall, P. J., Rose, U. S. R., Lewis, W. J. and Tumlinson, J. H. (1995). How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. USA* 92: 4169-4174.

Van Loon, J.J.A. and Dicke, M. (2000). Sensory ecology of arthropods utilizing plant infochemicals. In *Sensory Ecology*, eds. F.G. Barth and A. Schmid, pp. 253-270. Springer Verlag, Heidelberg.

Van Loon, J.J.A., De Boer, J.G. and Dicke, M. (2000a). Parasitoid-plant mutualism: parasitoid attack of herbivore increases plant reproduction. *Entomol. Exp. Appl.* 97: 219-227.

Van Loon, J.J.A., De Vos, E.W. and Dicke, M. (2000b). Orientation behaviour of the predatory hemipteran *Perillus bioculatus* to plant and prey odours. *Entomol. Exp. Appl.* 96: 51-58.

Vet, L. E. M. and Dicke, M. (1992). Ecology of infochemical use by natural enemies in a tritrophic context. *Ann. Rev. Entomol.* 37: 141-172.

Visser, J. H. and Avé, D. A. (1978). General green leaf volatiles in the olfactory orientation of the Colorado beetle, *Leptinotarsa decemlineata. Entomol. Exp. Appl.* 24: 538-549.

Wäckers, F.L. and Wunderlin, R. (1999). Induction of cotton extrafloral nectar production in response to herbivory does not require a herbivore-specific elicitor. *Entomol. Exp. Appl.* 91: 149-154.

Weber, H., Vick, B.A. and Farmer, E.E. (1997). Dinor-oxo-phytodienoic acid: A new hexadecanoid signal in the jasmonate family. *Proc. Natl. Acad. Sci. USA* 94: 10473-10478.

Whitman, D. W. and Eller, F. J. (1992). Orientation of *Microplitis croceipes* (Hymenoptera: Braconidae) to green leaf volatiles: Dose-response curves. *J. Chem. Ecol.* 18: 1743-1753.

Whitham, S.A. and Dinesh-Kumar, S.P. (2002). Signalling in plant-virus interactions. In *Plant Signal Transduction*, Scheel D. and Wasternack C. (eds). Oxford University Press, Oxford, UK, pp. 226-249.

Wildon, D. C., Thain, J. F., Minchin, P. E. H., Gubb, I. R., Reilly, A. J., Skipper, Y. D., Doherty, H. M., O'Donell, P. J. and Bowles, D. J. (1992). Electrical signalling and systemic proteinase inhibitor induction in the wounded plant. *Nature* 360: 62-65.

Wiskerke, J. S. C. and Vet, L. E. M. (1994). Foraging for solitarily and gregariously feeding caterpillars: a comparison of two related parasitoid species. *J. Insect. Beh.* 7: 585-603.

Yip, W.K., Moore, T. and Yang, S.F. (1992). Differential accumulation of transcripts for four tomato 1-aminocyclopropane-1-carboxylate synthase homologs under various conditions. *Proc. Natl. Acad. Sci. USA* 89: 2475-2479.

chapter

3

Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*:

chemical, behavioural, and gene-expression analysis

Remco M. P. van Poecke, Maarten A. Posthumus, and Marcel Dicke

" En toen op een mooie zondagmorgen de zon
stralend en warm opging
kroop uit het eitje - knak! - een hongerige rups "

Eric Carle - Rupsje Nooitgenoeg

Chapter 3

HERBIVORE-INDUCED VOLATILE PRODUCTION BY *Arabidopsis thaliana* LEADS TO ATTRACTION OF THE PARASITOID *Cotesia rubecula*: CHEMICAL, BEHAVIOURAL AND GENE-EXPRESSION ANALYSIS

Abstract

Many plant species are known to defend themselves against herbivorous insects indirectly, by producing volatiles in response to herbivory. These volatiles attract carnivorous enemies of the herbivores. Research on the model plant *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) has contributed considerably to the unraveling of signal transduction pathways involved in *direct* plant defence mechanisms against pathogens. Here we demonstrate that *Arabidopsis* is also a good candidate for studying signal transduction pathways involved in *indirect* defence mechanisms by showing that:

1) Adult females of *Cotesia rubecula* (Hymenoptera: Braconidae), a specialist parasitic wasp of *Pieris rapae* caterpillars (Lepidoptera: Pieridae), were attracted to *P. rapae*-infested *Arabidopsis* plants.
2) *Arabidopsis* plants infested by *P. rapae* emit volatiles from several major biosynthetic pathways, including terpenoids and green leaf volatiles. The blends of volatiles from herbivore infested and artificially damaged plants are quite similar. However, differences can be found with respect to a few components of the blend, such as two nitriles and the monoterpene myrcene, that were produced exclusively by caterpillar-infested plants, and methyl salicylate, that was produced in larger amounts by caterpillar-infested plants.
3) Genes from major biosynthetic pathways involved in volatile production were induced by caterpillar feeding. These include *AtTPS10*, encoding a terpene synthase involved in myrcene production, *AtPAL1*, encoding phenylalanine ammonia-lyase involved in methyl salicylate production, and *AtLOX2* and *AtHPL*, encoding lipoxygenase and hydroperoxide lyase respectively, both involved in the production of green leaf volatiles. *AtAOS*, encoding allene oxide synthase, involved in the production of jasmonic acid, was induced by herbivory as well.

---

1. Introduction

To defend themselves against herbivorous insects, plants can use both direct and indirect mechanisms (Karban and Baldwin, 1997; Dicke, 1999). Direct defence mechanisms have a direct effect on the herbivore, e.g. by negatively affecting the physiology of the herbivore, using toxic or antinutritional compounds, or by interfering with the behaviour of the herbivore, using repelling or deterring compounds (Karban and Baldwin, 1997). Indirect defence mechanisms promote the effectiveness of natural enemies of the herbivore, i.e. predators or parasitoids. One way of promoting the effectiveness of natural enemies is by producing volatiles in response to herbivory that can attract predators or parasitoids. This kind of tritrophic interaction has been shown for many, mainly agriculturally important, plant species, belonging to at least twelve plant families, in combination with a diverse range of herbivore and natural enemy species (Dicke, 1999).

Among the volatiles induced by herbivory, terpenoids, lipoxygenase-derived volatiles and methyl salicylate are commonly found. These compounds are produced via different biosynthetic pathways.

Terpenoids are a large and diverse group of compounds, present in most plant species (Gershenzon and Croteau, 1991). Although the early steps in terpenoid production can be divided in a cytosolic and a plastidic pathway (Lichtenthaler, 1999), products from both pathways are subsequently used by terpene synthases (TPS) (Bohlmann *et al.*, 1998; Bohlmann *et al.*, 2000), to produce mono-, sesqui-, and diterpenes.

The lipoxygenase-derived or “green leaf” volatiles are also common in plants. Important enzymes involved in their biosynthesis are lipoxygenase (LOX; Bell and Mullet, 1993) and hydroperoxide lyase (HPL, Bate *et al.*, 1998). Another branch of the lipoxygenase pathway produces the plant hormone jasmonic acid, which is involved in the induction of direct (Karban and Baldwin, 1997; McConn *et al.*, 1997) and indirect (Hopke *et al.*, 1994; Dicke *et al.*; 1999; Gols *et al.*, 1999; Thaler, 1999) defence mechanisms. For the production of jasmonic acid, the products from LOX are used by the enzyme allene oxide synthase (AOS) instead of HPL (Laudert *et al.*, 1996).

Methyl salicylate is a volatile derivative of the plant hormone salicylic acid (Lee *et al.*, 1995). The role of salicylic acid in direct defence mechanisms of plants against pathogens has been extensively reviewed (e.g. Dempsey *et al.*, 1999). Methyl salicylate is produced via the phenyl propanoid pathway. A key enzyme in the phenyl propanoid pathway is phenylalanine ammonia-lyase (PAL) (Lee *et al.*, 1995; Mauch-Mani and Slusarenko, 1996). Besides (methyl) salicylate, there are many other products from this pathway like lignin and flavonoids (Dixon and Paiva, 1995).

Signal-transduction pathways involved in herbivore-induced plant volatiles have only recently been studied (e.g. Hopke *et al.*, 1994; Paré and Tumlinson, 1997;

Boland *et al.*, 1999; Dicke *et al.*, 1999). However, there is a wealth of knowledge on signal transduction pathways in direct defences against both insects and pathogens (e.g. Karban and Baldwin, 1997; Pieterse *et al.*, 1998). A large part of the knowledge has been obtained by taking a molecular genetic approach (e.g. Delaney *et al.*, 1994). The incorporation of the model plant of molecular genetics, *Arabidopsis thaliana*, has contributed enormously to the progress in this field (e.g. Dietrich *et al.*, 1994; Pieterse *et al.*, 1998; Thomma *et al.*, 1998).

To date, only a few studies on insect-plant interactions have used *Arabidopsis* as a host plant (Grant-Petterson and Renwick, 1996; Mauricio and Rausher, 1997; McConn *et al.*, 1997; Mauricio, 1998; Reymond *et al.*, 2000; van Loon *et al.*, 2000; Stotz *et al.*, 2000), and only one of those focussed on indirect defences (van Loon *et al.*, 2000). Yet, *Arabidopsis* is known to be consumed by insects, such as caterpillars of *Pieris rapae* (Yano and Ohsaki, 1993; van Loon *et al.*, 2000). *Pieris rapae* caterpillars are hosts to parasitoids such as *Cotesia rubecula* (Agelopoulos and Keller, 1994; Geervliet *et al.*, 1994). Several crucifers are known to produce herbivore-induced volatiles in response to *P. rapae* damage and *C. rubecula* is attracted to these (Blaakmeer *et al.*, 1994; Geervliet *et al.*, 1994, 1997).

Taken together this indicates that *Arabidopsis* might be a good candidate for studying signal-transduction in indirect defences in plants. In the present study we investigate (1) whether *Arabidopsis* produces volatiles, induced by *P. rapae* feeding, that attract *C. rubecula* parasitoids, (2) what the composition is of the herbivore-induced volatile blend, and (3) whether genes from major biosynthetic pathways involved in the production of the identified volatiles are induced by caterpillar feeding.

2. Materials and Methods

2.1 Plants.

Arabidopsis thaliana (L.) Heynh., ecotype Columbia, was grown from seed in a greenhouse (20-30°C, 50-70% r.h., L8:D16). A few days prior to plant treatment, 9 to 10-week-old plants were transferred to a climate room (23±1°C, 50-70% r.h., 10 kLux, L8:D16). All plants used were in the vegetative state.

2.2 Insects

Pieris rapae was reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Icarus) in a climate room (21±1°C, 50-70% r.h., 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% r.h., L16:D8). Every year a fresh colony was initiated with individuals collected from fields near Wageningen, The Netherlands. For bioassay experiments, *C. rubecula* pupae were collected and transferred to a gauze cage in a climate room (23±1°C, 50-70% r.h., 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 ovipostion experience), are referred to as 'naive' wasps.

2.3 Plant treatments

For headspace collection and analysis, caterpillar-infested plants were obtained by placing twelve 1st instar *P. rapae* larvae on each plant. For the two choice bioassay and gene expression analysis, caterpillar-infested plants were obtained by placing two 1st instar *P. rapae* larvae on each leaf with a main vain longer than 3 cm, resulting on average in 24 larvae per plant. In both cases the larvae had fed for 24 hrs on the plants before these plants were used in experiments. Caterpillar-infested plants are referred to as plant-herbivore complex (PHC).

To obtain artificially damaged (AD) plants, all leaves with a main vein longer than 3 cm were rubbed with carborundum powder no. 180 (Cats Import, Hoogvliet, The Netherlands) on a moist cotton pad, just before an experiment (Mattiacci *et al.*, 1995).

Control (C) plants had not received any treatment but were of the same age and size as the treated plants and had been transferred to the climate room at the same time.

2.4 Bioassay

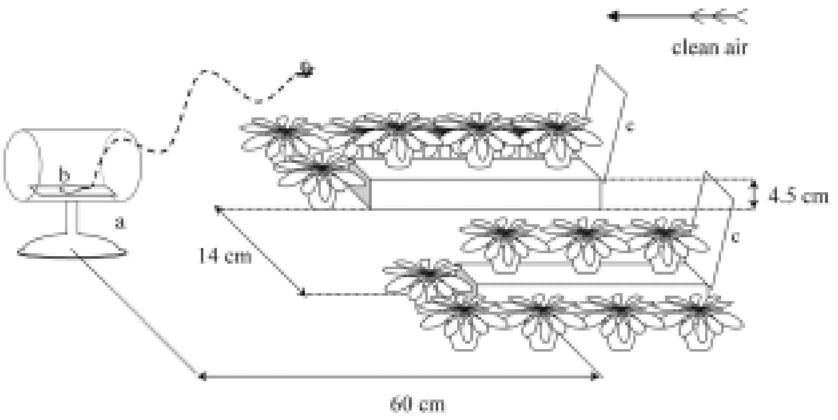
Parasitoid flight experiments were conducted in a windtunnel set-up (25±5°C, 50-70% r.h., 0.7 kLux) described by Geervliet *et al.* (1994), with a wind speed of 0.2 m/s.

For two-choice flight experiments, two odour sources were placed at the upwind end of the windtunnel. Each odour source consisted of 8 *Arabidopsis* plants, all receiving the same treatment. These 8 plants were placed in a 2x4 arrangement, with the 3 plants closest to the center and upwind end of the windtunnel on a 4.5 cm elevation (fig. 1). At the upwind end of each odour source, on the elevation, an 11x19 cm clear plexiglass rectangle was placed, to create some air turbulence. The distance between the odour sources was 14 cm.

One day before a bioassay, 4-to-6-days-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 caterpillar-damaged *Arabidopsis*, from which the caterpillars had been removed. The wasp plus leaf plus slide were transported to the middle of the release cylinder in the windtunnel, which was 60 cm downwind from the odour sources (fig. 1).

The flight behaviour of the 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 windtunnel besides the release cylinder or odour sources were recorded as '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 removed.

All combinations of the 3 odour sources (PHC-C, AD-C, PHC-AD) were tested on the same day. Per combination of odour sources, positions were changed from left to right and vice versa, to compensate for unforeseen asymmetric effects. In



Fihvsf 2. Windtunnel set-up. In the windtunnel, two odour sources, each consisting of 8 *Arabidopsis* plants were placed 60 cm upwind of the release cylinder (a). Of each odour source, 3 plants were placed on a 4.5 cm elevation. The distance between the two odour sources was 14 cm. Wasps were transported to the release cylinder on a microscope slide with one leaf from a caterpillar-damaged *Arabidopsis* from which the caterpillars had been removed (b). Clean laminar air was blown over the odour sources towards the release cylinder, at a speed of 0.2 m/s. Plexiglass rectangles (c) were placed at the upwind end of the odour sources to create some turbulence.

total, 50 wasps in 6 days were tested per odour combination.

Choices between two odour sources in the bioassay were statistically analysed using a chi-square test. Differences in the percentage of wasps making a 'choice' (responsiveness) between the 3 combinations of odour sources were tested using a contingency table on the absolute numbers.

For the no-choice flight experiment one odour source was placed at the upwind end of the windtunnel. The odour source consisted of one *Arabidopsis* plant placed on a 4.5 cm elevation. At the upwind end of the odour source, on the elevation, an 11x19 cm clear plexiglass rectangle was placed, to create some air turbulence. Selection and handling of the wasps was the same as for the two-choice experiments. On one day, the behaviour of 10 female wasps was subsequently recorded. Only flights that resulted in the first landing on the odour source were recorded as 'successful landing'. Landings on other parts of the windtunnel besides the release cylinder or odour sources were recorded as 'non-successful landing'. If the wasp remained in/on the release cylinder for longer than 20 min this was also recorded as 'non-successful landing'. After a 'successful' or 'non-successful landing' the wasp was removed.

On day 0 a single, undamaged *Arabidopsis* plant was used as odour source, and the behaviour of the 10 female wasps was recorded. After this test a single first instar *P. rapae* caterpillar that had just hatched was placed on the *Arabidopsis* plant. After 1, 2, 3, 4 and 6 days this plant was used as odour source for the bioassay. Every day, the behaviour of a new set of 10 females was recorded. This experiment was repeated 3 times. After pooling the data for every infestation period, differences between the number of wasps making a 'successful landing' between the subsequent days was tested using a contingency table. A Spearman's rank correlation test was used to analyse whether more days of feeding resulted in greater attraction.

2.5 Headspace collection and analysis

For dynamic headspace collection, performed in a climate room (23±1°C, 50-70% r.h., 10 kLux), 40 plants per treatment were used. The soil was removed from the roots by rinsing with tap water and subsequently the plants were placed in a 5 l glass jar on wet filter paper. The jar was closed with a glass lid having an air-inlet and air-outlet. In between lid and jar was a viton O-ring and the lid was tightly closed with a metal clamp. Pressurized air was filtered over silica gel, a molecular sieve, and activated charcoal. The resulting cleaned air was led through the jar with plants for 6 hrs, at a flow rate of 240 ml/min. To collect the headspace, the airstream was filtered at the outlet using 90 mg Tenax-TA in a glass tube. Subsequently, the Tenax tube was closed with 1/4" Swagelok caps and transferred to the GC-MS for analysis.

The collected volatiles were released from the Tenax using a Thermodesorption Cold Trap set-up (Chrompack, Middelburg, The Netherlands) as described by Mattiacci *et al* (1995), and transferred to the analytical column (Supelcowax 10 or Rtx-200, 60 m x 0.25 mm i.d., 0.25µm film thickness) of a gas chromatograph. The initial linear velocity of the helium carrier gas was 22 cm/s. The temperature of the column was raised from 40°C to 270°C at 4°C/min. The volatiles were analysed using a mass spectrometer (Finnigan MAT 95) and identified by comparison of the mass spectra with those in the NIST98 library and in the Wageningen Mass Spectral Database of Natural Products and by checking the retention index, unless stated otherwise.

Headspace collections of control (C) plants were carried out simultaneously, in parallel, with each of the two treatments (AD and PHC), to minimize variation among plant batches and day-to-day variation. The collections were made in triplicate.

When comparing damaged (either AD or PHC) with undamaged control (C) plants, a compound was considered to be emitted in larger amounts, if that compound showed larger peak areas in all three replicates of damaged plants, compared with the three replicates of undamaged control plants, or if that compound showed larger peak areas in two of the three replicates of damaged plants, the peak area of the third (and smallest) peak area being equal to the largest peak area of the three replicates of undamaged control plants. The latter occurred mostly when the compound was not detected in any of the three replicates of undamaged control plants and detected in two of the three replicates of damaged plants.

2.6 Gene expression analysis

For RNA isolation, the protocol described by Chang *et al.* (1993) was followed. Leaf material from 5-6 plants was pooled to obtain a total of 5 g. The protocol was adjusted to these 5 g. Isolated RNA was DNase treated (DNase I from GibcoBRL) using an RNase inhibitor (RNaseOUT from GibcoBRL) to preserve RNA quality. The concentration of total RNA was spectrophotometrically determined.

From the total RNA, 10 µg was separated on a formaldehyde-agarose gel, blotted on a nylon membrane (Hybond-N+, Amersham) (Sambrook *et al.*, 1989) and UV cross-linked. These Northern blots were prehybridized and hybridized at 65°C in hybridisation buffer (Church and Gilbert, 1984). The blots were washed for 15 min under medium stringency conditions (1 wash with 2x SSC, 0.1% SDS at rT, 1 wash with 2xSSC, 0.1% SDS at 55°C).

All probes were made using the RadPrime DNA Labeling System (GibcoBRL). Templates for the probes were obtained by PCR from cDNA clones (*AtTPS03, AtTPS10, AtLOX2, AtAOS, AtHPL*) or by RT-PCR from *Arabidopsis* total RNA (*AtPAL1, AtPAL2, AtPAL3*). Plasmids containing full-length cDNA’s of *AtTPS03* and *AtTPS10* were kindly provided by Jörg Bohlmann (see Bohlmann *et al.*, 2000), the plasmids containing cDNA’s of *AtLOX2, AtAOS* and *AtHPL* were described by Bell and Mullet (1993), Laudert *et al.* (1996), and Bate *et al.* (1998), respectively. Primers used for PCR were 5’-TAT-ACG-CGA-CCG-CCC-TTG-AG-3’ and 5’-GGC-GGA-GCT-TCG-AAC-TAC-AGA-G corresponding to bases 172827-172846 and 174843-174864 of *AtTPS03* (GenBank Accession No. Z97341); 5’-TTC-CGA-CTT-TTC-AGG-CAA-CAT-3’ and 5’-GAG-AGC-TGG-TCG-GAG-AAT-ACG-3’ corresponding to bases 327-347 and 1247-1267 of *AtTPS10* (GenBank Accession No. AF178535); 5’-GGA-TCG-ATA-AGA-CCG-CAG-AAC-ATG-GAG-3’ and 5’-GCT-GTC-GAC-TCA-TAG-AAC-TCT-TTT-AAG-G-3’ corresponding to bases 1969-1994 and 2364-2391 of *AtLOX2* (Bell and Mullet, 1993; underlined parts do not correspond to the plasmid template); 5’-CTT-TTC-ACC-GGT-ACT-TAC-ATG-CCG-3’ and 5’-GAT-AAC-CGT-AAA-GCA-TCT-CAC-CG-3’ corresponding to bases 447-470 and 1274-1291 of *AtAOS* (Laudert *et al.*, 1996); 5’-TCA-CAG-CTT-CCC-CTC-CGT-ACA-ATG-3’ and 5’-CCG-GAG-TCA-CCA-GTA-ATC-GTA-TCA-3’ corresponding to bases 147-170 and 1487-1510 of *AtHPL* (Bate *et al.*, 1998); 5’-AAG-AGC-AAC-GGA-GGA-GGA-GTG-G-3’ and 5’-GTG-GCG-GAG-TGT-GGC-AAT-GT-3’ corresponding to bases 150-171 and 603-622 of *AtPAL1* (Ohl *et al.*, 1990); 5’-AAC-GGC-ACC-GCA-TTA-CAA-ACA-GAA-3’ and 5’-AGA-TCC-AAC-CGC-CGT-GCC-ATT-A-3’ corresponding to bases 1838-1861 and 2455-2476 of *AtPAL2* (GenBank Accession no. L33678); and AGC-GAG-TGG-AGG-AGG-ACC-GAC-AGT-3’ and 5’-GGG-CGC-CCG-ATG-AGG-AAT-3’ corresponding to bases 2117-2140 and 3242-3259 of *AtPAL3* (GenBank Accession no. L33679). Ubiquitin expression served as a constitutive control.

For first strand cDNA synthesis, 10 µg of total RNA was used together with M-MLV Reverse Transcriptase, Random Primers and RNaseOUT (GibcoBRL). One twentieth of the cDNA mixture was used for subsequent PCR reactions with *Taq* DNA polymerase (GibcoBRL).

All mRNA analyses were done in triplicate, using new sets of plants.

3. Results

3.1 Bioassay

Cotesia rubecula females clearly preferred volatiles from *Pieris rapae*-infested (plant-herbivore complex, PHC) or artificially damaged (AD) *Arabidopsis* plants to those from undamaged plants (C). The wasps preferred odours from PHC to AD (fig. 2). The percentage of wasps making a choice did not differ significantly between the PHC-C (66%), AD-C (70%), or PHC-AD (82%) combinations (contingency table, P = 0.17).

The two-choice test was performed using 8 *Arabidopsis* plants per odour source with, in the case of PHC, on average 24 first instar *P. rapae* caterpillars. To test whether a single *Arabidopsis* plant damaged by a single *P. rapae* caterpillar also attracted *C. rubecula* females, we performed a no-choice test (fig. 3). The results show that a single plant infested by a single caterpillar attracts the parasitoid. The number of attracted wasps differed significantly between the different days (contingency table, P < 0.001). More days of feeding resulted in more attractive plants (Spearman's rank correlation test, P < 0.0025).

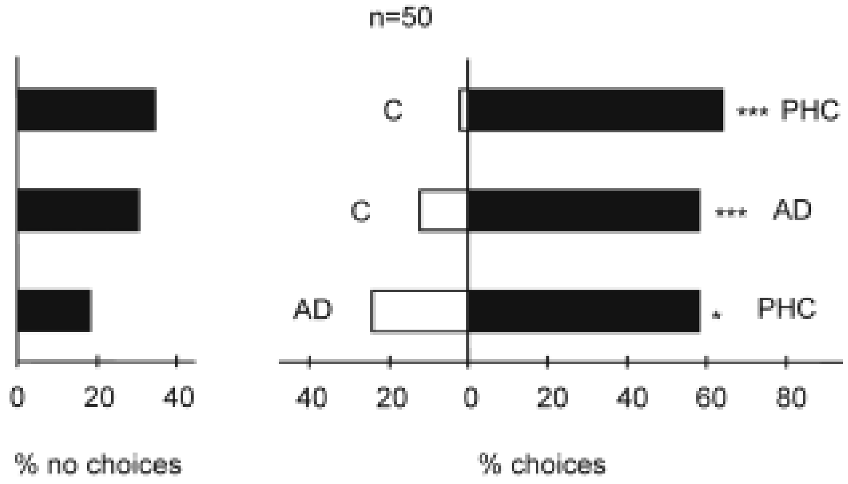
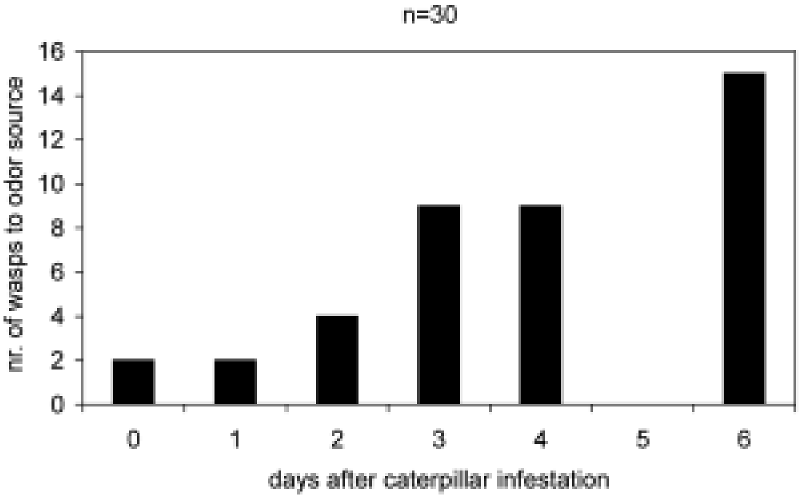


Figure 3. Responses of *C. rubecula* to herbivore-infested (PHC), artificially damaged (AD), and undamaged (C) *Arabidopsis* in a two-choice setup. Asterisks indicate a significant difference within a choice test: *** P<0.001, *P<0.05 (Chi^2). The left part of figure 2 shows the percentage of wasps that did not land on one of the two odor sources, for each odor combination. The right part of figure 2 shows the percentages of wasps landing either odor source, also for each odor combination. In total, 50 wasps were tested per combination.

3.2 Headspace analysis

In total 38 compounds derived from very different biosynthetic pathways were identified in the headspace of *Arabidopsis* plants (fig. 4). These comprise methyl salicylate, terpenoids, green leaf volatiles, sulfides, nitriles, alcohols, aldehydes, ketones and two cycloheptadienes.

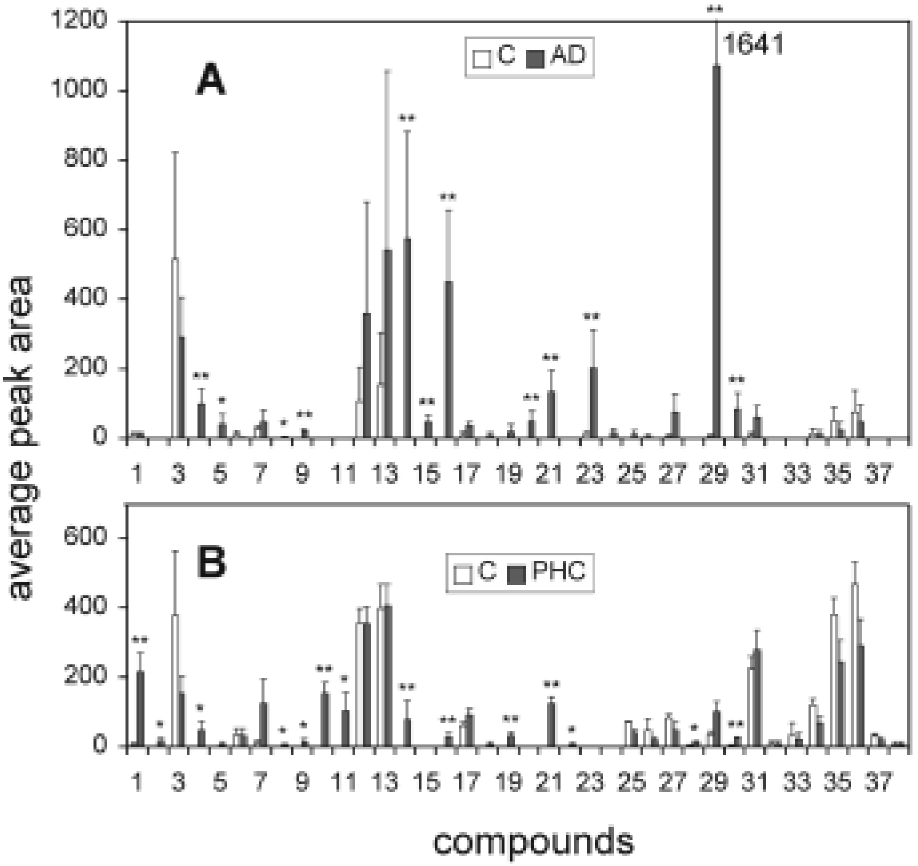


Fihvsf 4. Response of *C. rubecula* to a single herbivore-infested Arabidopsis plant in a no-choice set-up. At day 0 an undamaged Arabidopsis plant was tested for attractiveness to *C. rubecula* females. Subsequently, a single *P. rapae* larvae that had just hatched was placed on the plant. After 1, 2, 3, 4, and 6 days of infestation, the plant was tested again for attractiveness. In total, 30 wasps were tested in 3 replicate experiments per infestation period.

Artificially damaged plants emitted larger amounts of volatiles than undamaged plants (fig. 4A). Artificially damaged plants clearly emitted more green leaf volatiles ((Z)-3-hexen-1-ol (nr. 14), (Z)-3-hexen-1-yl acetate (nr. 20), and 1-hexanol (nr. 16)) than undamaged plants. Emission of terpenoids (β-ionone (nr. 4) and β-cyclocitral (nr. 5)), sulfides (dimethyl disulfide (nr. 8) and dimethyl trisulfide (nr. 9)), alcohols (3-pentanol (nr. 20), 1-penten-3-ol (nr. 21) and 2-ethyl-1-hexanol (nr. 23)) and ketones (3-pentanone (nr. 29) and 1-penten-3-one (nr. 30)) was also induced by artificial damage (fig. 4A).

Caterpillar-infested plants emitted slightly larger amounts of volatiles than undamaged plants (fig. 4B). The caterpillar-infested plants emitted more methyl salicylate (nr.1), terpenoids (myrcene (nr. 2) and β-ionone (nr. 4)), sulfides (dimethyl disulfide (nr. 8) and dimethyl trisulfide (nr. 9)), nitriles (5-(methylthio)-pentanenitrile (nr. 10) and 6,7-dithiaoctanenitrile (nr. 11)), green leaf volatiles ((Z)-3-hexen-1-ol (nr. 14) and 1-hexanol (nr. 16)), some alcohols (1-pentanol (nr. 19), 1-penten-3-ol (nr. 21), and 2-penten-1-ol (nr. 22), and ketones (1-penten-3-one and 2-pentanone) than undamaged plants (fig. 4B). In fact, the monoterpene myrcene (nr. 2), the two nitriles (nr. 10 and 11) and the alcohol 2-penten-1-ol (nr. 22) were exclusively found in the volatile blend of caterpillar-infested plants. Methyl salicylate (nr. 1), the two nitriles (nr. 10 and 11), the alcohol 1-pentanol (nr. 19) and the ketone 2-pentanone (nr. 28) were induced by caterpillar feeding (Fig. 4B) but not by artificial damage (Fig 4A). The green leaf volatile (Z)-3-hexen-1-yl acetate (nr. 15) and the alcohol 3-pentanol (nr. 20) were only found in the blend of artificially damaged plants. In contrast to herbivore-infested plants, artificially damaged plants showed induced emission of the terpenoid c-cyclocitral (nr. 5), the alcohol 2-ethyl-1-hexanol (23) and the ketone 3-pentanone (29). Two remarkable compounds that made up a large part of the blend emitted by undamaged, artificially damaged and caterpillar-infested plants were tentatively identified as 6-

[(*Z*)-1-butenyl]-1,4-cycloheptadiene (nr. 12; ectocarpene) and 6-butyl-1,4-cycloheptadiene (nr. 13; dictyotene), based on mass spectra.



Fihvsf 5. Headspace analysis of herbivore-infested (PHC), artificially damaged (AD), and undamaged (C) *Arabidopsis*. Average peak areas of three replicates are shown. Error bars indicate the standard error. Compounds marked with ** showed larger peak areas in all three replicates of treated plants (either AD or PHC), compared with the three replicates of undamaged control plants (C). Compounds marked with * showed larger peak areas in two of the three replicates of treated plants, the peak area of the third (and smallest) peak area being equal to the largest peak area of the three replicates of undamaged control plants.

Compound nrs are:

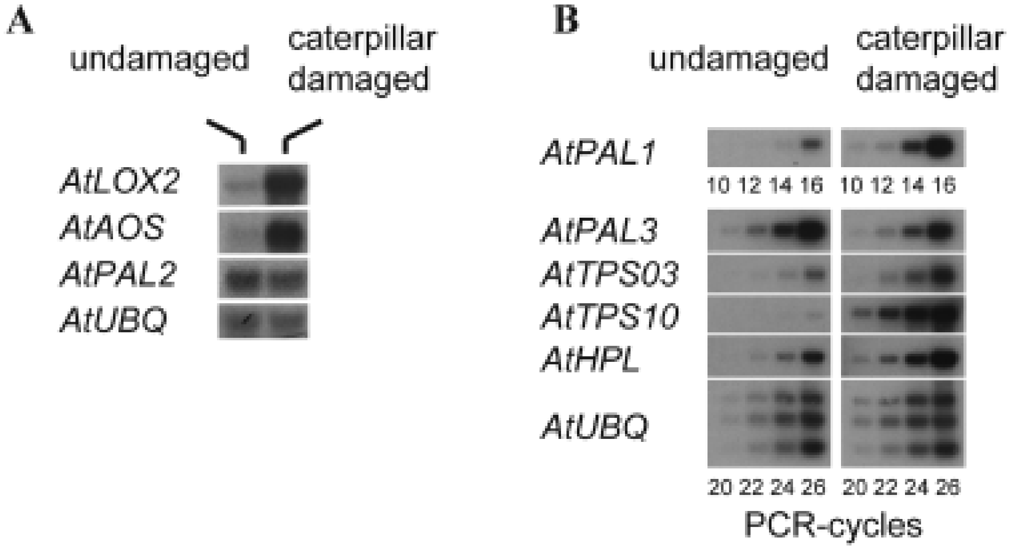
(1) methyl salicylate, (2) myrcene, (3) limonene, (4) c-ionone,
(5) c-cyclocitral, (6) longifolene, (7) (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene,
(8) dimethyldisulfide, (9) dimethyltrisulfide, (10) 5-(methylthio) pentanenitrile,
(11) 6,7-dithiooctanenitrile, (12) 6-[(*Z*)-1-butenyl]-1,4-cycloheptadiene,
(13) 6-butyl-1,4-cycloheptadiene, (14) (*Z*)-3-hexen-1-ol, (15) (*Z*)-3-hexen-1-yl acetate,
(16) 1-hexanol, (17) hexanal, (18) 1-butanol, (19) 1-pentanol, (20) 3-pentanol,
21) 1-penten-3-ol, (22) 2-penten-1-ol, (23) 2-ethyl-1-hexanol, (24) 1-octen-3-ol,
(25) 1-nonanol, (26) 1-dodecanol, (27) 1-tetradecanol, (28) 2-pentanone,
(29) 3-pentanone, (30) 1-penten-3-one, (31) 4-methyl-3-heptanone,
(32) 4-methyl-4-hepten-3-one, (33) heptanal, (34) octanal, (35) nonanal, (36) decanal,
(37) undecanal, (38) 1-nonene.

3.3 Gene expression analysis

Using Northern blot analysis the expression of some genes (*AtLOX2, AtAOS,* and *AtPAL2*) could be detected. For other genes (*AtPAL1*, *AtPAL3*, *AtTPS03*, *AtTPS10*, and *AtHPL*) the Northern blot analysis resulted in very weak or undetectable radioactivity signals (data not shown). In those cases, RT-PCR was used. Gene expression was considered to be induced by *P. rapae* feeding if all three replicates from caterpillar damaged plants showed higher expression than the three replicates from undamaged plants.

Expression of three genes from the lipoxygenase pathway, *AtLOX2* (Bell and Mullet, 1993), *AtHPL* (Bate *et al.*, 1998) and *AtAOS* (Laudert *et al.*, 1996), was induced by caterpillar feeding (fig. 5, A and B). The expression of two genes involved in terpenoid biosynthesis, was examined: *AtTPS03* and *AtTPS10*. Expression of the genes *AtTPS03*, a novel monoterpene synthase (Bohlmann, unpublished results), and *AtTPS10*, a myrcene / ocimene synthase (Bohlmann *et al.*, 2000), was also induced by 24 h of caterpillar feeding (fig 5B).

In *Arabidopsis*, three genes encoding for phenylalanine ammonia-lyase, an enzyme involved in the production of methyl salicylate, have been isolated: *AtPAL1* (Ohl *et al.*, 1990), *AtPAL2*, and *AtPAL3* (Wanner *et al.*, 1995). *AtPAL1* was clearly induced by caterpillar feeding, whereas *AtPAL2* and *AtPAL3* were not induced (fig. 5, A and B)



Fihvsf 6. Induction of gene expression by herbivore damage. RNA was extracted from undamaged *Arabidopsis* plants or *Arabidopsis* plants that had been subjected to 24 hrs of *Pieris rapae* caterpillar feeding. For Northern analysis (fig. 4A), five micrograms of RNA was loaded in each lane. Northern blot analysis was performed for *AtLOX2, AtAOS,* and *AtPAL2*. RT-PCR analysis, and subsequent blotting and radioactive labeling, was performed for *AtPAL1* (10, 12, 14, and 16 PCR-cycles), *AtPAL3, AtTPS03, AtTPS10,* and *AtHPL* (20, 22, 24, and 26 PCR-cycles) (fig. 4B). Ubiquitin expression served as a constitutive control, both for Northern and RT-PCR (20, 22, 24, and 26 PCR-cycles) analysis. Note that autoradiography films were exposed differently for different genes, therefore comparison of expression levels between genes is not possible.

4. Discussion and conclusions

4.1 Discussion

The attraction of carnivorous arthropods by plants in response to herbivory has been intensively studied over the past 15 years (Turlings *et al.*, 1995; Dicke, 1999). This phenomenon has been shown for a large number of plants in various families, including the Brassicaceae (Dicke, 1999). Our data show that *Arabidopsis thaliana* plants are able to do so as well. Damage results in the production of volatiles that attract *C. rubecula*, a specialist parasitoid of *Pieris rapae.* The parasitoids prefer volatiles from caterpillar-infested or mechanically damaged plants to those from undamaged plants. They also distinguish between artificially damaged and caterpillar-infested plants, preferring the latter (fig. 2). This resembles the findings for agricultural plants in the Brassicaceae family with the same herbivore and parasitoid species (Agelopoulos and Keller, 1994; Geervliet *et al.*, 1994). The difference in attractiveness between artificially damaged and caterpillar-infested plants cannot be explained by a response to odours emitted by the caterpillars themselves or their by-products. *Pieris rapae* larvae or their by-products are only slightly attractive to *C. rubecula* females (Geervliet et al. 1994). Moreover, the wasps do not distinguish between caterpillar-infested plants and previously infested plants from which the caterpillars and their by-products were removed 24 h after infestation (Geervliet *et al.*, 1994). Additionally, fig. 3 shows that feeding damage of only a single *P. rapae* caterpillar on a single *Arabidopsis* plant results in an increased attractiveness of this plant to *C. rubecula* females. This is before the larva reaches the third instar, so the biomass of the caterpillar is very small and feces production is at a relatively low level, as compared to later instars.

Recently it was demonstrated that the seed production of *Arabidopsis* plants, on which caterpillars of *P. rapae* are feeding that had been parasitised by *C. rubecula*, is similar to the seed production of undamaged plants and significantly greater than the seed production of plants fed upon by unparasitized caterpillars (van Loon *et al.*, 2000). This finding shows that *Arabidopsis*' fitness benefits greatly from the attraction of parasitoid wasps by means of herbivore-induced volatiles.

Both artificially damaged and caterpillar infested plants emit larger amounts of volatiles than undamaged plants (fig. 4). The volatile blend of damaged plants, either artificially or by caterpillar feeding, contains compounds that could not be detected in the blend of undamaged plants. The blends of artificially damaged and caterpillar-infested plants are quite similar, although some differences can be found. Artificially damaged plants showed induced emission of especially green leaf volatiles and ketones, but also of alcohols, terpenoids and sulfides. Caterpillar infested plants showed induced emissions of especially methyl salicylate and nitriles, but also of alcohols, ketones, sulfides and terpenoids. The nitriles and the terpenoid myrcene were not detected in the blends of artificially damaged plants and undamaged plants. Methyl salicylate (Dicke *et al.*, 1990; 1998; C.E.M. van den Boom, in prep.) and terpenoids (Dicke *et al.*, 1990; 1998; Turlings *et al.*, 1990; Loughrin *et al.*, 1994) are known to be induced by herbivory in many plant species. Both methyl salicylate and several terpenoids are attractants for predatory mites (Dicke *et al.*, 1990). Moreover, terpenoids are likely to be involved in the attraction of parasitoids (Turlings *et al.*, 1991). Nitriles are induced by spider mite feeding on cucumber (Takabayashi *et al.*, 1994) and on apple (Takabayashi *et al.*, 1991). However, in cruciferous plants like Brussels sprouts (Blaakmeer *et al.*, 1994), red and white cabbage (Geervliet *et al.*, 1997), methyl salicylate, terpenoids, and nitriles are hardly or not induced by herbivory. The induction of methyl salicylate, the terpenoids myrcene and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and the nitriles 5-(methylthio)pentanenitrile and 6,7-dithiooctanenitrile in *Arabidopsis* by *P. rapae* infestation is, therefore, noteworthy.

In all plant species studied so far, artificial damage leads to the emission of green leaf volatiles (e.g. Dicke *et al.*, 1990; Turlings *et al.*, 1990; Whitman and Eller, 1990; Takabayashi *et al.*, 1991). It is, therefore, not surprising that green leaf volatiles are also present in the volatile blends of all caterpillar-infested plant species tested so far (e.g. Whitman and Eller, 1990; Turlings *et al.*, 1990), now also including *Arabidopsis*. In Brussels sprouts and red and white cabbage they are highly induced by caterpillar feeding, forming the largest part of the volatile blends (Blaakmeer *et al.*, 1994; Mattiacci *et al.*, 1995; Geervliet *et al.*, 1997). Females of the parasitoid wasps *Microplitis croceipes* and *Netelia heroica* are attracted to individual green leaf volatiles (Whitman and Eller, 1990).

The presence of the two compounds tentatively identified as the cycloheptadienes ectocarpene and dictyotene in the volatile blend of *Arabidopsis* is remarkable, as these compounds are not common in higher plants. They are mostly known as sex pheromones in algae (Boland, 1995). Yet they have been found in some higher plants, e.g. in *Senecio isatideus* (Bohlmann *et al.*, 1979). In *Arabidopsis*, these compounds are not induced by caterpillar feeding.

The emission of volatiles after herbivory can be a passive release of stored compounds but can also result from an increased production of volatiles. Using isotope labelling techniques, Paré and Tumlinson (1997) showed that in response to herbivory, cotton produces some volatiles *de novo* and others not. In Lima bean and cucumber the activity of a terpenoid-producing enzyme is induced by herbivore damage (Bouwmeester *et al.*, 1999). Here, we studied the expression of genes involved in early (*AtLOX*, *AtPAL1*, *AtPAL2* and *AtPAL3*) or late (*AtTPS03* and *AtTPS10*, *AtHPL*) steps of several biosynthetic pathways of volatiles emitted by *Arabidopsis* (fig. 5). Gene expression levels correlated well with the emission of induced volatiles.

Recently, Bohlmann and coworkers reported the isolation of a terpene synthase from *Arabidopsis*, *AtTPS10*. Functional characterization showed that the AtTPS10 protein is a myrcene / ocimene synthase. Myrcene is the major product of the enzyme, ocimene is produced in lower amounts (Bohlmann *et al.*, 2000). Both myrcene emission and *AtTPS10* gene expression are induced by 24 h of caterpillar feeding, indicating that herbivory leads to induced myrcene production. Expression of a novel monoterpene synthase gene, *AtTPS03* (Bohlmann, unpublished results), was also induced by caterpillar feeding (fig. 5B).

In *Arabidopsis* three PAL genes have been identified (Wanner *et al.*, 1995). Mauch-Mani and Slusarenko (1996) have shown that a major function of PAL in *Arabidopsis* is the production of salicylic acid. As methyl salicylate is a derivative of salicylic acid (Lee *et al.*, 1995), it is likely that induced PAL gene expression can lead to increased production of methyl salicylate. This is supported by our data, which show an induced *AtPAL1* expression after caterpillar feeding (fig. 5B) and an increased emission of methyl salicylate. Expression of the other two known genes encoding for PAL in *Arabidopsis*, *AtPAL2* and *AtPAL3*, was not induced. Possibly, these two genes are involved in other processes within the phenyl propanoid pathway than production of methyl salicylate. Similar expression patterns were reported by Reymond *et al.* (2000 and on www.unil.ch/ibpv) after 3 hrs of *P. rapae* feeding on *Arabidopsis*, although they found a weak induction of *AtPAL3*.

Important genes in the production of green leaf volatiles like *AtLOX2* (Bell and Mullet, 1993) and *AtHPL* (Bate *et al.*, 1998) are both induced in caterpillar-infested *Arabidopsis* (fig. 5, A and B). However, the products formed by lipoxygenase are also used by allene oxide synthase (AOS), involved in jasmonic acid production. Expression of the *AtAOS* gene in *Arabidopsis* is also induced by caterpillar feeding (fig. 5A). Therefore we cannot predict whether increased *AtLOX* and *AtHPL* expression will lead to increased green leaf volatile production, increased jasmonic acid production or both. Jasmonic acid is an important elicitor involved in induced indirect defences of plants. In *Vicia faba* (Blechert *et al.*, 1995) and tobacco (McCloud and Baldwin, 1997) caterpillar feeding resulted in increased endogenous jasmonic acid levels. Jasmonic acid treatment induces volatile emissions in several plant species (Boland et al., 1995) including Lima bean (Hopke *et al.*, 1994; Dicke *et al.*, 1999), maize (Hopke *et al*, 1994), and gerbera (Gols *et al.*, 1999), resulting in the attraction of carnivores (Dicke *et al.*, 1999; Gols *et al.*, 1999) and leads to higher parasitism rates of caterpillars feeding on tomato (Thaler, 1999). Expression of *AtLOX2*, *AtAOS*, and *AtHPL* is already induced after 3 hrs of *P. rapae* feeding on *Arabidopsis* (Reymond *et al.*, 2000).

To our knowledge, no genes involved in the production of nitriles have been identified in *Arabidopsis* so far.

The herbivory-induced expression of genes involved in volatile production indicates that *Arabidopsis* is actively producing volatiles after herbivory. Moreover, the differences in attraction of *C. rubecula* and the composition of the volatile blends of artificially damaged and caterpillar-infested plants suggest that herbivory leads to the production of *C. rubecula* attracting volatiles distinct from the volatiles emitted after artificial damage. However, mimicking the mechanical aspects of caterpillar damage correctly is difficult if not impossible. Expression of *AtLOX2* (Bell and Mullet, 1993), *AtHPL* (Bate *et al.*, 1998), *AtAOS* (Laudert *et al.*, 1996), and *AtPAL1* (Mizutani *et al.*, 1997) is also induced by artificial damage. Further experiments are needed and currently undertaken to investigate whether *Arabidopsis* indeed produces volatiles that are specific for herbivory compared to mechanical damage.

4.2 Conclusions

This study on indirect plant defence combines behavioural and chemical analyses with gene expression analysis. Our data show that *Pieris rapae*-infested *Arabidopsis thaliana* attract *Cotesia rubecula* parasitoids. Caterpillar infested plants release volatiles from several major biosynthetic pathways and genes involved in the production of these volatiles are induced by herbivory. Many mutants and transgenic lines of *Arabidopsis* are available, that are altered in signal transduction pathways of direct defence mechanisms or in the biosynthesis of secondary plant metabolites. In addition, the complete genome of *Arabidopsis* will be sequenced soon and the knowledge of direct defence mechanisms is steadily increasing (e.g. Pieterse and van Loon, 1999). Therefore, *Arabidopsis* constitutes an interesting tool to study signal transduction of induced indirect defence in plants.

Acknowledgments

We thank Maarten Koornneef, Kees van Loon, Corné Pieterse, and Joop van Loon for their advise on this research and valuable comments on the manuscript, Herman Dijkman, Leo Koopman, Frans van Aggelen and Andre Gidding for the insect rearing, Casper Pillen for rearing of *Arabidopsis* plants, Jörg Bohlmann and Jonathan Gershenzon for the *AtTPS03* and *AtTPS10* plasmids, Steven Rothstein, Jocelyne Le Tarte and Richard Stahl for providing the *AtLOX2, AtAOS* and *AtHPL* plasmids, and the PhD discussion group of the Laboratory of Entomology for comments on this manuscript. M.D. was partly funded by the Uyttenboogaart-Eliasen Foundation, Amsterdam.

References

Agelopoulos, N.G., Keller, M.A. 1994. Plant-natural enemy association in the tritrophic system, *Cotesia rubecula-Pieris rapae*-Brassicaceae (Crucifera): II. Preference of *C. rubecula* for landing and searching. *J Chem Ecol* 20: 1735-1748

Alborn, T., Turlings, T.C.J., Jones, T.H., Steinhagen, G., Loughrin, J.H., Tumlinson, J.H. 1997. An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276: 945-949

Bate, N.J., Sivasankar, S., Moxon, C., Riley, J.M.C., Thompson, J.E., Rothstein, S.J. 1998. Molecular characterization of an *Arabidopsis* gene encoding hydroperoxide lyase, a cytochrome p-450 that is wound inducible. *Plant Physiol* 117: 1393-1400

Bell, E., Mullet, J.E. 1993. Characterization of an *Arabidopsis* lipoxygenase gene responsive to methyl jasmonate and wounding. *Plant Physiol* 103: 1133-1137

Blaakmeer, A., Geervliet, J.B.F., Loon, J.J.A., Posthumus, M.A., van Beek, T.A., de Groot, AE 1994. Comparative headspace analysis of cabbage plants damaged by two species of Pieris caterpillars: consequences for in-flight host location by Cotesia parasitoids. *Entomol Exp Appl* 73: 175-182

Blechert, S., Brodschelm, W., Holder, S., Kammerer, L., Kutchan, T.M., Mueller, M.J., Xia, Z.Q., Zenk, M.H. 1995. The octadecanoic pathway: signal molecules for the regulation of secondary pathways. *Proc Natl Acad Sci USA* 92: 4099-4105

Bohlmann, J., Meyer-Gauen, G., Croteau, R. 1998. Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proc Natl Acad Sci USA* 95: 4126-4133

Bohlmann, J., Martin, D., Oldham, N.J., Gershenzon, J. 2000) Terpenoid secondary metabolism in *Arabidopsis thaliana*: cDNA cloning, characterization and functional expression of a myrcene/ocimene synthase. *Arch Biochem Biophys* 375: 261-269

Bohlmann, F., Zdero, C., Berger, D., Suwita, A., Mahanta, P., Jeffrey, C. 1979. Neue furanoeremophilane und weitere inhaltsstoffe aus Südafrikanischen *Senecio*-arten. *Phytochemistry* 18: 79-93

Boland, W. 1995. The chemistry of gamete attraction: Chemical structures, biosynthesis, and (a)biotic degradation of algal pheromones. *Proc Natl Acad Sci USA* 92: 37-43

Boland, W., Hopke, J., Donath, J., Nüske, J., Bublitz, F. 1995. Jasmonic acid and coronatin induce odour production in plants. *Angew Chem* 34:1600-1602.

Boland, W., Koch, T., Krumm, T., Piel, J., Jux, A. 1999. Induced biosynthesis of insect semiochemicals in plants. In: D.J. Chadwick and J.A. Goode (eds), *Insect-plant interactions and induced plant defence*. John Wiley & Sons, NY, pp 110-131

Bouwmeester, H.J., Verstappen, F.W.A., Posthumus, M.A., Dicke, M. 1999. Spider mite-induced (3*S*)-(*E*)-neridol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. *Plant Physiol* 121: 173-180

Chang, S., Puryear, J., Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol reporter* 11: 113-116

Church, G., Gilbert, W. 1984. Genomic sequencing. *Proc Natl Acad Sci USA* 92: 4114-4119

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gutrella, M., Kessmann, H., Ward, E., Ryals, J. 1994. A central role of salicylic acid in plant disease resistance. *Science* 266: 1247-1250

Dempsey, D.A., Shah, J., Klessig, D.F. 1999. Salicylic acid and disease resistance in plants. *Crit Rev Plant* Sci 18: 547-575

Dicke, M. 1999. Evolution of induced indirect defence of plants. In: R Tollrian and CJ Harvell (eds), *The ecology and evolution of inducible defences*. Princeton University Press, Princeton, New Jersey, pp 62-88

Dicke, M., Vet, L.E.M. 1999. Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore. In: H. Olff, V.K Brown, and R.H. Drent (eds), *Herbivores: between plants and predators*, Blackwell Science, Oxford, UK, pp. 483-520

Dicke, M., van Beek, T.A., Posthumus, M.A., Ben Dom, N., van Bokhoven, H., de Groot, AE. 1990. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *J Chem Ecol* 16: 381-396

Dicke, M., Gols, R., Ludeking, D., Posthumus, M.A. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in Lima bean plants. *J Chem Ecol* 25: 1907-1922

Dicke, M., Takabayashi, J., Posthumus, M.A., Schutte, C., Krips, O.E. 1998. Plant-phytoseiid interactions mediated by herbivore-induced plant volatiles: variation in production of cues and in response of predatory mites. *Exp Appl Acar* 22: 311-333

Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A., Dangl, J.L. 1994. *Arabidopsis* mutants simulating disease resistance response. *Cell* 77: 565-577

Dixon, R.A., Paiva, N.L. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* 7: 1085-1097

Geervliet, J.B.F., Vet, L.E.M., Dicke, M. 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomol Exp Appl* 73: 289-297

Geervliet, J.B.F., Posthumus, M.A., Vet, L.E.M., Dicke, M. 1997. Comparative analysis of headspace volatiles from different caterpillar-infested or uninfested food plants of *Pieris* species. *J Chem Ecol* 23: 2935-2954

Gershenzon, J., Croteau, R. 1991. Terpenoids. In G.A. Rosenthal and M.R. Berenbaum (eds), *Herbivores: their interactions with secondary plant metabolites.*,Vol 1. Academic Press, New York, pp 165-219

Gols, R., Posthumus, M.A., Dicke, M. 1999. Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent *Phytoseiulus persimilis*. *Entomol Exp Appl* 93: 77-86

Grant-Petersson, J., Renwick, J.A.A. 1996. Effects of ultraviolet-B exposure of *Arabidopsis thaliana* on herbivory by two crucifer-feeding insects (Lepidoptera). *Environ Entomol* 25: 135-142

Hopke, J., Donath, J., Blechert, S., Boland, W. 1994. Herbivore-induced volatiles: the emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by a c-glucosidase and jasmonic acid. *FEBS Letters* 352: 146-150

Karban, R., Baldwin, I.T. 1997. *Induced Responses to Herbivory*. University of Chicago Press Ltd., Chicago

Laudert, D., Pfannschmidt, U., Lottspeich, F., Holländer-Czytko, H., Weiler, E.W. 1996. Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. *Plant Mol Biol* 31: 323-335

Lee, H.L., Léon, J., Raskin, I. 1995. Biosynthesis and metabolism of salicylic acid. *Proc Natl Acad Sci USA* 92: 4076-4079

Lichtenthaler, H.K. 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annu Rev Plant Physiol Plant Mol Biol* 50: 47-65

Loughrin, J.H., Manukian, A., Heath, R.R., Turlings, T.C.J., Tumlinson, J.H. 1994. Diurnal cycle of emission of induced volatile terpenoids herbivore-injured cotton plants. *Proc Natl Acad Sci USA* 91: 11836-11840

Mattiacci, L., Dicke, M., Posthumus, M.A. 1995. c-Glucosidase: an elicitor of herbivore-induced plant odour that attracts host-searching parasitic wasps. *Proc Natl Acad Sci USA* 92: 2036-2040

Mauch-Mani, B., Slusarenko, A.J. 1996. Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *Plant Cell* 8: 203-212

Mauricio, R. 1998. Costs of resistance to natural enemies in field populations of the annual plant *Arabidopsis thaliana*. *American Naturalist* 151: 20-28

Mauricio, R., Rausher, M.D. 1997. Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defence. *Evolution* 5: 1435-1444

Mizutani, M., Ohta, D., Sato, R. 1997. Isolation of a cDNA and a genomic clone encoding cinnamate-4-hydroxylase from *Arabidopsis* and its expression manner in planta. *Plant Physiol* 113: 755-763

McCloud, E.S., Baldwin, I.T. 1997. Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*. *Planta* 203: 430-435

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., Browse, J. 1997. Jamonate is essential for insect defence in *Arabidopsis*. *Proc Natl Acad Sci USA* 94: 5473-5477

Ohl, S., Hedrick, S.A., Chory, J., Lamb, J.C. 1990. Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. *Plant Cell* 2: 837-848

Paré, P.W., Tumlinson, J.H. 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol* 114: 1161-1167

Pieterse, C.M.J., van Loon, L.C. 1999. Salicylic acid-independent plant defence pathways. *Trends Plant Sci* 4: 52-58

Pieterse, C.M.J., van Wees, S.C.M., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., van Loon, L.C. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* 10: 1571-1580

Reymond, P., Weber, H., Damond, M., Farmer, E.E. 2000. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell* 12: 707-719.

Sabelis, M.W., van Baalen, M., Bakker, F.M., Bruin, J., Drukker, B., Egas, M., Janssen, A.R.M., Lesna, I.K., Pels, B., van Rijn, P.C.J., Scutareanu, P. 1999. The evolution of direct and indirect plant defence against herbivorous arthropods. In: H. Olff, V.K. Brown, and R.H. Drent (eds), *Herbivores: between plants and predators*. Blackwell Science Ltd, Oxford, pp 109-166

Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, New York

Stotz, H.U., Pittendrigh, B.R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A., Mitchell-Olds, T. 2000. Induced plant defence responses against chewing insects. Ethylene signaling reduces resistance of *Arabidopsis* against Egyptian cotton worm but not diamondback moth. *Plant*

Physiol. 124:1007-1017.

Takabayashi, J., Dicke, M., Posthumus, M.A. 1991. Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: relative influence of plant and herbivore. *Chemoecology* 2: 1-6

Takabayashi, J., Dicke, M., Posthumus, M.A. 1994. Volatile herbivore-induced terpenoids in plant-mite interactions: Variation caused by biotic and abiotic factors. *J Chem Ecol* 20: 1329-1354

Thaler, J.S. 1999. Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399: 686-687

Thomma, B.P.H.J., Eggermont, K., Pennickx, I.A.M.A., Mauch-Mani, B.M., Vogelsang, R., Cammue, B.P.A., Broekaert, W.F. 1998. Separate jasmonate-dependent and salicylate-dependent defence-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc Natl Acad Sci USA* 95: 15107-15111

Turlings, T.C.J., Tumlinson, J.H., Lewis, W.J. 1990. Exploitation of herbivore-induced plant odours by host-seeking parasitic wasps. *Science* 250: 1251-1253

Turlings, T.C.J., Tumlinson, J.H., Heath, R.R., Proveaux, A.T., Doolittle, R.E. 1991. Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *J Chem Ecol* 17: 2235-2251

Turlings, T.C.J., McCall, P., Alborn, H.T., Tumlinson, J.H. 1993. An elicitor in caterpillar oral secretions that induces corn seedlings to emit chemical signals attractive to parasitic wasps. *J Chem Ecol* 19: 411-425

Turlings, T.C.J., Loughrin, J.H., McCallm P.J., Rösem U.S.R., Lewis, W.J., Tumlinson, J.H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc Natl Acad Sci USA* 92: 4169-4174

Van Loon, J.J.A., de Boer, J.G., Dicke, M. 2000. Parasitoid-plant mutualism: parasitoid attack of herbivore increases plant reproduction. *Entomol Exp Appl* 97:219-227

Wanner, L.A., Li, G., Ware, D., Somssich, I.E., Davis, K.R. 1995. The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. *Plant Mol Biol* 27: 327-338

Whitman, D.W., Eller, F.J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1: 69-75

Yano, S., Ohsaki, N. 1993. The phenology and intrinsic quality of wild crucifers that determine the community structure of their herbivorous insects. *Researches on Population Ecology* 35: 151-170

chapter

4

Induced parasitoid attraction by *Arabidopsis thaliana*:

involvement of the octadecanoid and the salicylic acid pathway

Remco M.P. van Poecke and Marcel Dicke

"It was the hormones talking, I hoped"

Richard Asplin - T-shirt and genes

Chapter 4

INDUCED PARASITOID ATTRACTION BY *Arabidopsis thaliana*: INVOLVEMENT OF THE OCTADECANOID AND THE SALICYLIC ACID PATHWAY

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 to 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.

---

1. 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 (see Chapter 2).

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, 1999ab). As in direct defences, indirect defences can be constitutively present or inducible (Chapter 2). 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 Loon *et al.*, 2000; Fritzsche-Hoballah and Turlings, 2001). 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 indicated that both the octadecanoid and the salicylic acid pathway play an important role in the attraction of carnivores (Chapter 2).

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 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 (e.g. 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, we have demonstrated that *Arabidopsis* can be used for studying indirect plant defences: *Arabidopsis* attracts parasitoid wasps (*Cotesia rubecula*) upon infestation with *Pieris rapae* caterpillars (Chapter 3). 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 exogenous application of JA or JA-like compounds (Chapter 2), 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. Our 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*.

2. Materials and methods

2.1 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% r.h., L8:D16). A few days prior to the experiments, 8 to 10-week-old plants were transferred to a climate room (23±1°C, 50-70% r.h., 10 kLux, L8:D16). All plants used were in the vegetative state.

2.2 Insects

Pieris rapae was reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Icarus) in a climate room (21±1°C, 50-70% r.h., 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% r.h., L16:D8). For bioassays, *C. rubecula* pupae were collected and transferred to a gauze cage in a climate room (23±1°C, 50-70% r.h., 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.

2.3 Plant treatments

Caterpillar-infested plants were obtained by placing 1st 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.

2.4 Bioassay

Parasitoid two-choice flight experiments were conducted in a wind tunnel set-up (25±5°C, 50-70% r.h., 0.7 kLux) described by Geervliet *et al.* (1994), that was modified according to van Poecke *et al.* (Chapter 3), 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 8 *Arabidopsis* plants, all having received the same treatment. One day before a bioassay, 4 to 7-days-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 4 instead of 8 *Arabidopsis* plants. The female wasps were released from a glass vial (Ø 1.5 cm, length 5 cm), after a 10 min experience with 3 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 ‘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.

2.5 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 1st 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.

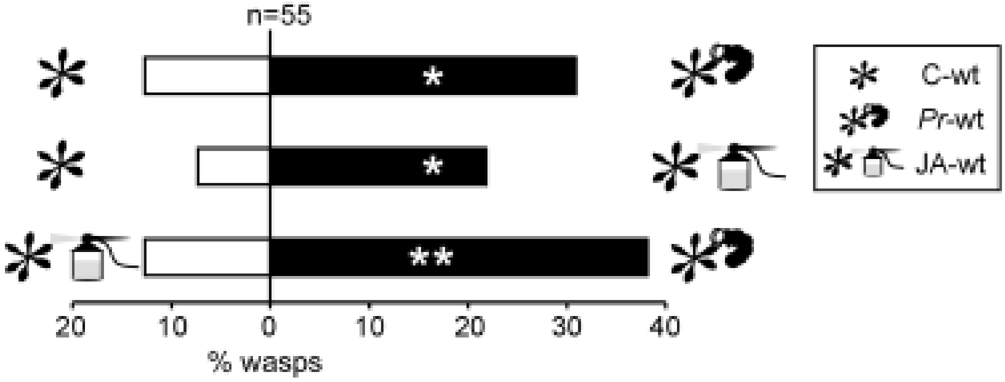


Figure 1. Response of *C. rubecula* to jasmonic acid-treated wild-type *Arabidopsis* compared to *P. rapae*-infested plants. In a two-choice set-up the response of naive *C. rubecula* females was tested to undamaged wild-type plants (C-wt), wild-type plants infested with 15 first instar *P. rapae* caterpillars 24 hrs before an experiment (*Pr*-wt), and wild-type plants treated with a 1 mM jasmonic acid solution 24 hrs 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: ** $P<0.01$, * $P<0.05$ (X^2-test).

3. Results

3.1 Influence of the octadecanoid pathway on parasitoid behaviour

To test the effect of JA treatment on the attractiveness of *Arabidopsis* plants towards parasitoid wasps, we sprayed wild-type *Arabidopsis* plants 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 we have demonstrated previously (Chapter 3). 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, we also studied the influence of the octadecanoid pathway on parasitoid behaviour 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 ($P=4*10^{-7}$; Fig. 2).

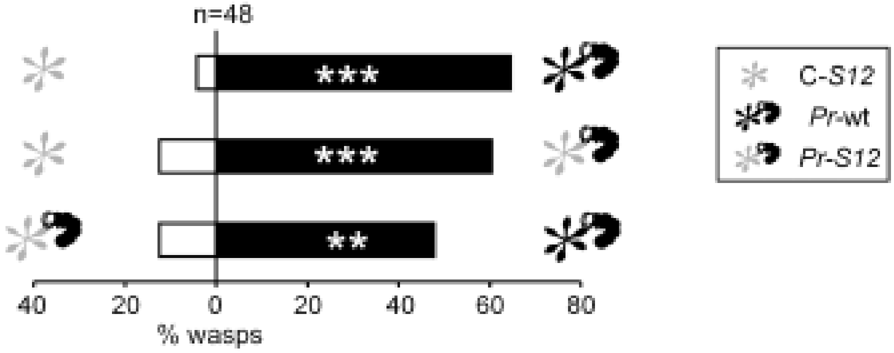


Figure 2. Response of *C. rubecula* to *P. rapae*-infested wild-type and *S12* transgenic *Arabidopsis.* In a two-choice set-up the response of naive *C. rubecula* females was tested to undamaged *S12* plants (C-*S12*), wild-type plants infested with 5 first instar *P. rapae* caterpillars 24 hrs before an experiment (*Pr*-wt), and *S12* plants infested with 5 first instar *P. rapae* caterpillars 24 hrs before an experiment (*Pr-S12*). *S12* plants do not accumulate jasmonic acid after wounding. The percentage of in total 48 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: *** $P<0.001$, ** $P<0.01$ (X^2-test).

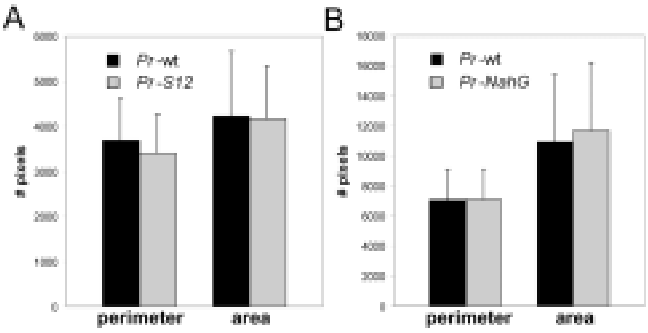


Figure 3. Damage analysis of *P. rapae*-infested wild-type, *S12* and *NahG* plants. Both the perimeter and the area of damage inflicted by 5 first instar *P. rapae* larvae after 24 hrs of feeding on a single wild-type or *S12* plant were determined for in total 40 wild-type and 40 *S12* plants (A). Similarly the damaged inflicted by 10 first instar *P. rapae* larvae after 24 hrs of feeding on a single wild-type or *NahG* plant was determined for in total 27 wild-type and 27 *NahG* plants (B). The average number of pixels with standard deviation is shown. There is no significant difference between wild-type and *S12* plants and between wild-type and *NahG* plants, both for the perimeter and area of damage (*P*>0.05, two-tailed Wilcoxon-Mann-Whitney ranking test).

3.2 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, we measured the damaged inflicted by five 1st instar caterpillars 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).

3.3 Influence of the salicylic acid pathway on parasitoid behaviour

Although SA treatment of plants induces many direct defence-related processes (Dempsey *et al.*, 1999), to our knowledge the effect of SA application on indirect defences has not been studied so far. Therefore, we tested the effect of spraying wild-type *Arabidopsis* plants with a 5 mM salicylic acid solution on the attraction of *C. rubecula.* 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 ($P=0.59$). *Pieris 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, we used *NahG* plants 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

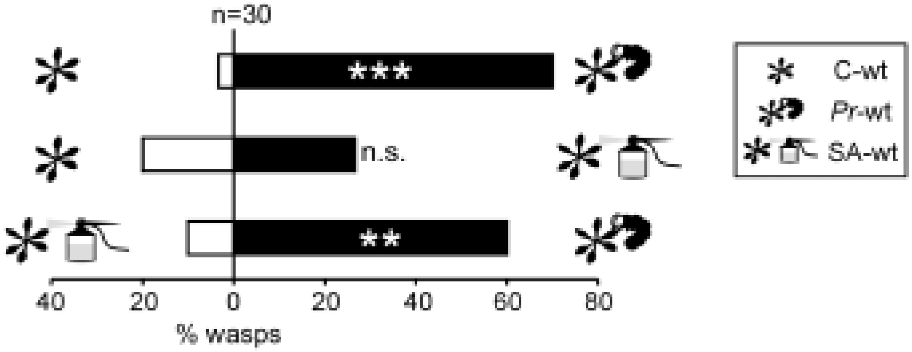


Figure 4. Response of *C. rubecula* to salicylic acid-treated wild-type *Arabidopsis* compared to *P. rapae*-infested plants. In a two-choice set-up the response of naive *C. rubecula* females was tested to undamaged wild-type plants (C-wt), wild-type plants infested with 20 first instar *P. rapae* caterpillars 24 hrs before an experiment (*Pr*-wt), and wild-type plants treated with a 5 mM salicylic acid solution 24 hrs before an experiment (SA-wt). The percentage of in total 28 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: *** $P<0.001$, ** $P<0.01$, n.s. = not significant (X^2-test).

over undamaged *NahG* plants ($P=2*10^{-5}$; Fig. 5). Similarly, *P. rapae*-infested wild-type plants were preferred over undamaged *NahG* plants ($P=3*10^{-6}$; Fig. 5).

3.4 Influence of salicylic acid on herbivore behaviour

Just as for the S-12 plants, we 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 ten 1st instar *P. rapae* larvae on *NahG* plants did not differ from the damage inflicted by an equal number of 1st instar larvae on wild-type plants, when

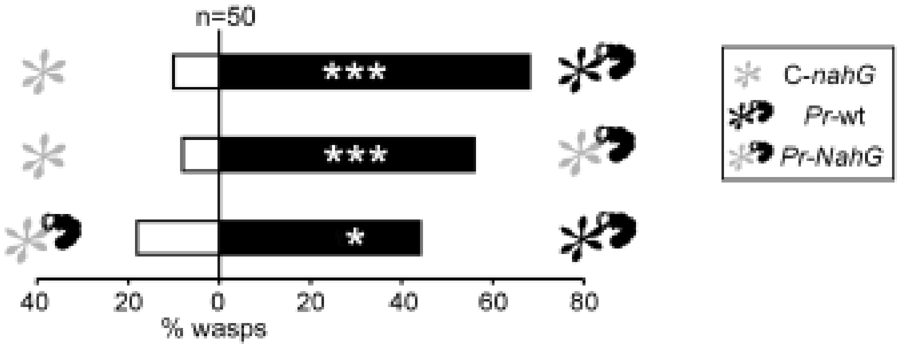


Figure 5. Response of *C. rubecula* to *P. rapae*-infested wild-type and *NahG* transgenic plants. In a two-choice set-up the response of naive *C. rubecula* females was tested to undamaged *NahG* plants (C-*NahG*), wild-type plants infested with 5 first instar *P. rapae* caterpillars 24 hrs before an experiment (*Pr*-wt), and *NahG* plants infested with 5 first instar *P. rapae* caterpillars 24 hrs before an experiment (*Pr-NahG*). *NahG* plants cannot accumulate salicylic acid. The percentage of in total 50 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: *** $P<0.001$, * $P<0.05$ (X^2-test).

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).

4. Discussion and conclusions

4.1 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* (e.g. 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 was 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 synthesised *de novo* (Pare 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 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 (Chapter 2).

Our 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 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* (Chapter 3). 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 croceipis* 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 (Chapter 2), including caterpillar-infested *Arabidopsis* (Chapter 3). 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 only JA 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.

4.2 Conclusions

This study clearly demonstrates that *Arabidopsis* is an excellent tool to study the signalling pathways involved in herbivory-induced parasitoid attraction. To get a better understanding of the role 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 to determine 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.

Acknowledgements

We thank Mara Roosjen, Ronald van Zoest and Joep Burger for their help on the damage analysis, Maarten Koornneef, Kees van Loon, Corné Pieterse, Martin de Vos, and the PhD discussion group of the Laboratory of Entomology for their valuable comments on the manuscript, Herman Dijkman, Leo Koopman, Frans van Aggelen and Andre Gidding for the insect rearing, and Casper Pillen for rearing of Arabidopsis plants. M.D. was partly funded by the Uyttenboogaart-Eliasen Foundation, Amsterdam.

References

Bell E, Creelman RA, Mullet JE. 1995. A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis. Proceedings of the National Academy of Sciences, USA* 92, 8675-8679.

Bi JL, Murphy JB, Felton GW. 1997. Does salicylic acid act as a signal in cotton for induced resistance to *Helicoverpa zea*? *Journal of Chemical Ecology* 23, 1805-1818.

Blechert S, Brodschelm W, Hölder S, Kammerer L, Kutchan TM, Mueller MJ, Xia ZQ, Zenk MH. 1995. The octadecanoic pathway: signal molecules for the regulation of secondary pathways. *Proceedings of the National Academy of Sciences, USA* 92, 4099-4105.

De Bruyne M, Dicke M, Tjallingii WF. 1991. Receptor cell responses in the anterior tarsi of *Phytoseiulus persimilis* to volatile kairomone components. *Experimental & Applied Acarology* 13, 53-58.

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gutrella M, Kessmann H, Ward E, Ryals J. 1994. A central role of salicylic acid in plant disease resistance. *Science* 266, 1247-1250.

Dempsey DA, Shah J, Klessig DF. 1999. Salicylic acid and disease resistance in plants. *Critical Reviews in Plant Sciences* 18, 547-575.

Dicke M. 1999a. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? *Entomologia Experimentalis et Applicata* 91, 131-142.

Dicke M. 1999b. Evolution of induced indirect defense of plants. In: Tollrian R and Harvell CD (eds), *The ecology and evolution of inducible defenses.* Princeton: Princeton University Press, pp 62-88.

Dicke M, Gols R, Ludeking D, Posthumus MA. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *Journal of Chemical Ecology* 25, 1907-1922.

Dicke M, van Beek TA, Posthumus MA, Ben Dom N, van Bokhoven H, de Groot AE. 1990. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *Journal of Chemical Ecology* 16, 381-396.

Felton GW, Korth KL, Bi JL, Wesley SV, Huhman DV, Mathews MC, Murphy JB, Lamb C, Dixon RA. 1999. Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. *Current Biology* 9, 317-320.

Fritzsche-Hoballah ME, Turlings TCJ. 2001. Experimental evidence that plants under caterpillar attack may benefit from attracting parasitoids. *Evolutionary Ecology Research* 3, 553-565.

Gardner HW. 1995. Biological roles and biochemistry of the lipoxygenase pathway. *Hortscience* 30, 197-205.

Geervliet JBF, Vet LEM, Dicke M. 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula. Entomologia Experimentalis et Applicata* 73, 289-297.

Halitschke R, Schittko U, Pohnert G, Boland W, Baldwin IT. 2001. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata.* III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiology* 125, 711-717.

Karban R, Baldwin IT. 1997. Induced Responses to Herbivory. The University of Chicago Press Ltd., Chicago, USA.

Kessler A, Baldwin IT. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291, 2141-2144.

Koch T, Krumm T, Jung V, Engelberth J, Boland W. 1999. Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. *Plant Physiology* 121, 153-162.

Mauricio R, Rausher MD. 1997. Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution* 51, 1435-1444.

McCloud ES, Baldwin IT. 1997. Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*. *Planta* 203, 430-435.

McConn M, Creelman RA, Bell E, Mullet JE, Browse J. 1997. Jasmonate is essential for insect defense *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* 94, 5473-5477.

Moran PJ, Thompson GA. 2001. Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. *Plant Physiology* 125, 1074-1085.

Niki T, Mitsuhara I, Seo S, Ohtsubo N, Ohashi Y. 1998. Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenensis-related (PR) protein genes in wounded mature tobacco leaves. *Plant and Cell Physiology* 39, 500-507.

Ozawa R, Arimura G, Takabayashi J, Shimoda T, Nishioka T. 2000. Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. *Plant and Cell Physiology* 41, 391-398.

Pare PW, Tumlinson JH. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiology* 121, 325-331.

Peña-Cortés H, Albrecht T, Prat S, Weiler EW, Willmitzer L. 1993. Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. *Planta* 191, 123-128.

Piel J, Atzorn R, Gäbler R, Kühnemann F, Boland W. 1997. Cellulysin from the plant parasitic fungus *Trichoderma viride* elicits volatile biosynthesis in higher plants via the octadecanoid signalling cascade. *FEBS Letters* 416, 143-148.

Pieterse CMJ, van Loon LC. 1999. Salicylic acid-independent plant defence pathways. *Trends in Plant Science* 4, 52-58.

Reinbothe S, Mollenhauer B, Reinbothe C. 1994. JIPs and RIPs: the regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. *Plant Cell* 6, 1197-1209.

Reymond P, Weber H, Damond M, Farmer EE. 2000. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell* 12, 707-719.

Stintzi A, Weber H, Reymond P, Browse J, Farmer EE. 2001. Pland defense in absence of jasmonic acid: The role of cyclopentenones. *Proceedings of the National Academy of Sciences, USA* 98, 12837-12842.

Stout MJ, Fidantsef AL, Duffey SS, Bostock RM. 1999. Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology* 54, 115-130.

Thaler JS. 1999. Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399, 686-687.

Thomma BPHJ, Eggermont K, Pennickx IAMA, Mauch-Mani BM, Vogelsang R, Cammue BPA, Broekaert WF. 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proceedings of the National Academy of Sciences, USA* 95, 15107-15111.

van Loon JJA, de Boer JG, Dicke M. 2000. Parasitoid-plant mutualism: parasitoid attack of herbivore increases plant reproduction. *Entomologia Experimentalis et Applicata* 97, 219-227.

Whitman DW, Eller FJ. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1, 69-75.

Xu Y, Chang PFL, Liu D, Narasimhan ML, Raghothama KG, Hasegawa PM, Bressan RA. 1994. Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell* 6, 1077-1085.

chapter 5

Signal transduction in induced volatile emission by *Arabidopsis*:

molecular genetic and chemical analysis of different genotypes

Remco M.P. van Poecke, Maarten A. Posthumus, and Marcel Dicke

"Back in those days everything was simpler and more confused."

Jim Morrison - Stoned immaculate

Chapter 5

SIGNAL TRANSDUCTION IN INDUCED VOLATILE EMISSION BY *Ara*ahcnorhr: MOLECULAR GENETIC AND CHEMICAL ANALYSIS OF DIFFERENT GENOTYPES

Abstract

Plants produce volatiles upon herbivory that can attract natural enemies of the herbivores, such as parasitoid wasps. Jasmonic acid (JA) and salicylic acid (SA) are important components in signal transduction resulting in herbivory-induced volatile production. Here, we take a molecular genetic approach to investigate the role of JA and SA in caterpillar-induced volatile production by Arabidopsis thaliana. We analysed the volatile blends emitted by Pieris rapae-infested wild-type Col0 and transgenic S-12 and NahG plants. LOX2-cosuppressed S-12 plants are unable to accumulate JA upon wounding, while NahG plants do not accumulate SA. Moreover, we studied the effect of JA-treatment on volatile emissions by wild-type plants.

Caterpillar-infested S-12 plants showed a reduced emission of all plant volatiles. However, JA-treatment induced the emission of specific compounds, mainly terpenoids and methyl salicylate (MeSA). Infested NahG plants did not emit MeSA and showed a reduced emission of the terpenoid (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. These data suggest an important role for MeSA and terpenoids as parasitoid attractants.

Additionally, we analysed the expression patterns of genes involved in the production of volatiles in undamaged and P. rape-infested S-12, NahG and wild-type plants. Two genes showed distinct expression patterns. As expected, expression of LOX2 was suppressed in S-12 plants. Caterpillar-induced expression of HPL was reduced in NahG plants, due to higher expression in undamaged NahG and lower expression in P. rapae-infested NahG.

Based on these data we propose a model on signal-transduction in caterpillar-induced volatile production in Arabidopsis through the concerted action of JA and SA.

---

1. Introduction

Upon herbivory, plants start to emit volatiles that can attract natural enemies of the herbivores, such as parasitoids (Turlings et al., 1995; Dicke, 1999a, b). By doing so, the plant promotes the effectiveness of these carnivores, which can be beneficial for both the carnivore and the plant (Dicke, 1999a; Van Loon et al., 2000; Fritzsche-Hoballah and Turlings, 2001). This increased attraction of carnivores constitutes an indirect plant defence that is widespread among plants (Dicke, 1999b). Like many direct plant defences, the increased emission of volatiles is the result of an increased production and such an inducibility of defences requires signal transduction leading from herbivore damage to volatile emissions (Karban and Baldwin, 1997; Pare and Tumlinson, 1997). Evidence has been accumulating that the octadecanoid pathway, which produces plant hormones such as 12-oxo-phytodienoic acid (OPDA) and jasmonic acid (JA), plays an important role in this signal transduction (Piel et al., 1997; Dicke et al., 1999; Koch et al., 1999; Kessler and Baldwin, 2001). Recently, an additional signalling role for salicylic acid (SA) has been suggested (Ozawa et al., 2000).

Most studies so far used exogenous application of elicitors or inhibitors and monitored volatile emission or the behavioural responses of the parasitoid/predator. The use of molecular genetic tools such as mutant or transgenic plants and cDNA microarrays has proven to be successful in unravelling direct defence mechanisms of plants, especially for the model plant Arabidopsis thaliana (McConn et al., 1997; Pieterse and van Loon, 1999; Reymond et al., 2000; Kessler and Baldwin, 2002). Recently, we have demonstrated that Arabidopsis can be used to study induced indirect defence against insects as well. Arabidopsis starts to emit volatiles upon herbivory by Pieris rapae caterpillars and these volatiles attract females of the parasitoid Cotesia rubecula. Moreover, we showed that genes that are likely to be involved in the induction or production of volatiles are induced by P. rapae feeding (Chapter 3). As in many other plant species (Dicke et al., 1990; Turlings et al., 1995; Geervliet et al., 1997; Pare and Tumlinson, 1997) the volatile blend of Arabidopsis consists of compounds such as green leaf volatiles, terpenoids, and methyl salicylate (Chapter 3).

Jasmonic acid-treatment of Arabidopsis wild-type Col0 resulted in an increased attraction of C. rubecula, whereas transgenic S-12 plants, that do not show wound-induced accumulation of JA because of cosuppression of the LOX2 gene (Bell et al., 1995), were less attractive when infested by P. rapae caterpillars compared to wild-type plants (Chapter 4). This demonstrated that the octadecanoid pathway is indeed involved in herbivory-induced parasitoid attraction by Arabidopsis. Pieris rapae-infested NahG plants, that cannot accumulate SA due to the expression of the bacterial salicylate hydroxylase gene NahG (Delaney et al., 1994), are also less attractive than P. rapae-infested wild-type plants (Chapter 4). However, it was unclear whether this was due to a signalling function of SA or because the reduced amounts of SA also resulted in reduced amounts of the volatile ester of SA, methyl salicylate (MeSA), which is known to attract carnivorous mites (Dicke et al., 1990) and is detected by chemoreceptors on the antennae of C. rubecula (H.M. Smid, *personal communication*).

Here, we present a chemical analysis of the volatile blends emitted by *P. rapae*-infested wild-type, S-12 and NahG plants and of the blend emitted by JA-treated wild-type *Arabidopsis*. The data demonstrate that JA and SA work synergistically in the signalling of herbivore-induced volatile emissions by *Arabidopsis*. In addition, we studied the expression patterns of genes that are likely to be involved in volatile production by *Arabidopsis*. These include genes from the octadecanoid pathway, involved in the production of OPDA, JA and green leaf volatiles (*LOX2*, *AOS*, and *HPL*), *PAL1* which is involved in the production of SA (and MeSA) and *TPS10* which is involved in the production of the monoterpene myrcene (Bell et al., 1995; Laudert and Weiler, 1998; Bate et al., 1998; Mauch-Mani and Slusarenko, 1996; Bohlmann et al., 2000). Expression patterns are shown for undamaged and *P. rapae*-infested wild-type, S-12 and NahG plants. This molecular genetic approach to herbivore-induced volatiles of *Arabidopsis* provides novel insights into the underlying signal transduction that are difficult to obtain by conventional approaches. A signal-transduction model is developed and discussed in the context of existing knowledge of signal transduction in other types of induced plant defence.

2. Materials and methods

2.1 Plants

Arabidopsis thaliana ecotype Columbia (Col-0), the transgenic NahG line (*Arabidopsis thaliana* ecotype Col-0 transformed with the bacterial salicylate hydroxylase encoding *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% r.h., L8:D16). A few days prior to the experiments, 8 to 10-week-old plants were transferred to a climate room (23±1°C, 50-70% r.h., 10 kLux, L8:D16). All plants used were in the vegetative state.

2.2 Insects

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

2.3 Plant treatments

Caterpillar-infested plants were obtained by placing ten (gene-expression analysis) or twenty (headspace collection) first instar *P. rapae* larvae on each plant. After the larvae had fed for 24 h on the plants, these plants were used in experiments. Wild-type, S-12, and NahG plants were of the same age.

Wild-type (Col-0) plants treated with jasmonic acid were sprayed in a group of 40 plants with in total 50 ml of a 1.0 mM (±)-jasmonic acid (Sigma-Aldrich) solution, 24 h before an experiment. At the same time, a group of 40 wild-type control plants was sprayed with water of the same pH (obtained by adding HCl) as the JA-solution.

2.4 Headspace collection and analysis

For dynamic headspace collection, performed in a climate room (23±1°C, 50-70% r.h., 10 kLux), 40 plants per treatment were used. The roots of *P. rapae*-infested wildtype, S-12, and NahG plants were rinsed with tap water to remove the soil just prior to headspace collection. Subsequently, the plants were placed on filter paper in three separate 5 *l* glass jars. The jars were closed with a glass lid having an air-inlet and air-outlet. In between lid and jar was a viton O-ring and the lid was tightly closed with a metal clamp. Pressurized air was filtered over silica gel, molecular sieve 4A and 13X, and activated charcoal. The resulting cleaned air was led through the jars with plants for 6 h, at a flow rate of 75 m*l* min^{-1} jar^{-1}. To collect the headspace, the airstream was filtered at the outlet using 90 mg Tenax-TA in a glass tube. Subsequently, the tube was closed with 1/4" Swagelok caps and transferred to the GC-MS for analysis.

To collect volatiles from plants treated with JA and control plants, they were cut from their roots just prior to volatile sampling. The 40 JA-treated and the 40 control plants were placed on moist filter paper in two separate 5 *l* glass jars. Subsequently, the plants were placed on filter paper in two separate 5 *l* glass jars. The volatiles were collected as described above, with a flow rate of 240 m*l* min^{-1} jar^{-1}.

The collected volatiles were released from the Tenax using a Thermodesorption Cold Trap set-up as described by Mattiacci et al (1995), and transferred to the analytical column (Supelcowax 10 or DB5, 60 m x 0.25 mm *i.d.*, 0.25µm film thickness) of a gas chromatograph. The initial linear velocity of the helium carrier gas was 22 cm s^{-1}. The temperature of the column was raised from 40°C to 270°C at 4°C min^{-1}. The volatiles were analysed using a mass spectrometer (Finnigan MAT 95) and identified by comparison of the mass spectra with those in the NIST98 library and in the Wageningen Mass Spectral Database of Natural Products and by checking the retention index, unless stated otherwise.

Headspace collections of JA-treated and control plants were carried out simultaneously, in parallel, to minimize variation among plant batches and day-to-day variation. Similarly, headspace collections of *P. rapae*-infested wild-type, S-12 and NahG plants were done simultaneously, in parallel. The collections of each treatment were made in triplicate. Only volatiles that were detected in at least two out of three replicates of the same treatment/genotype are shown. Both differences between the total amount of volatiles and the relative amount of each individual compound emitted by plants from different treatments/genotypes were tested using the Mann-Whitney U test.

2.5 Gene expression analysis

From 20 undamaged and 20 *P. rapae*-infested wild-type, S-12, and NahG plants, RNA was isolated using RNAwiz (Ambion Inc, Austin, USA). Leaf material was harvested from two batches of 10 plants per treatment, batch 1 being sown and harvested one week before batch 2. From the P. rapae-infested plants, one damaged leaf per plant was used. From undamaged plants, one leaf of similar size was used per plant. Per treatment the 20 leaves were pooled before RNA isolation. RNA isolation of the different treatments was done simultaneously. The concentration of total RNA was spectrophotometrically determined.

From the total RNA, 10 µg per treatment was used for cDNA synthesis. One twentieth of the cDNA mixture was used for subsequent PCR reactions. Primers as described in Chapter 3 were used for *LOX2*, *AOS*, *HPL*, *PAL1*, *TPS10*, and *UBQ*. PCR-products were used for southern hybridisation and radioactively labelled using a modified Church and Gilbert method (Amersham, Piscataway, USA). Radioactivity was quantified using a phosphoimager.

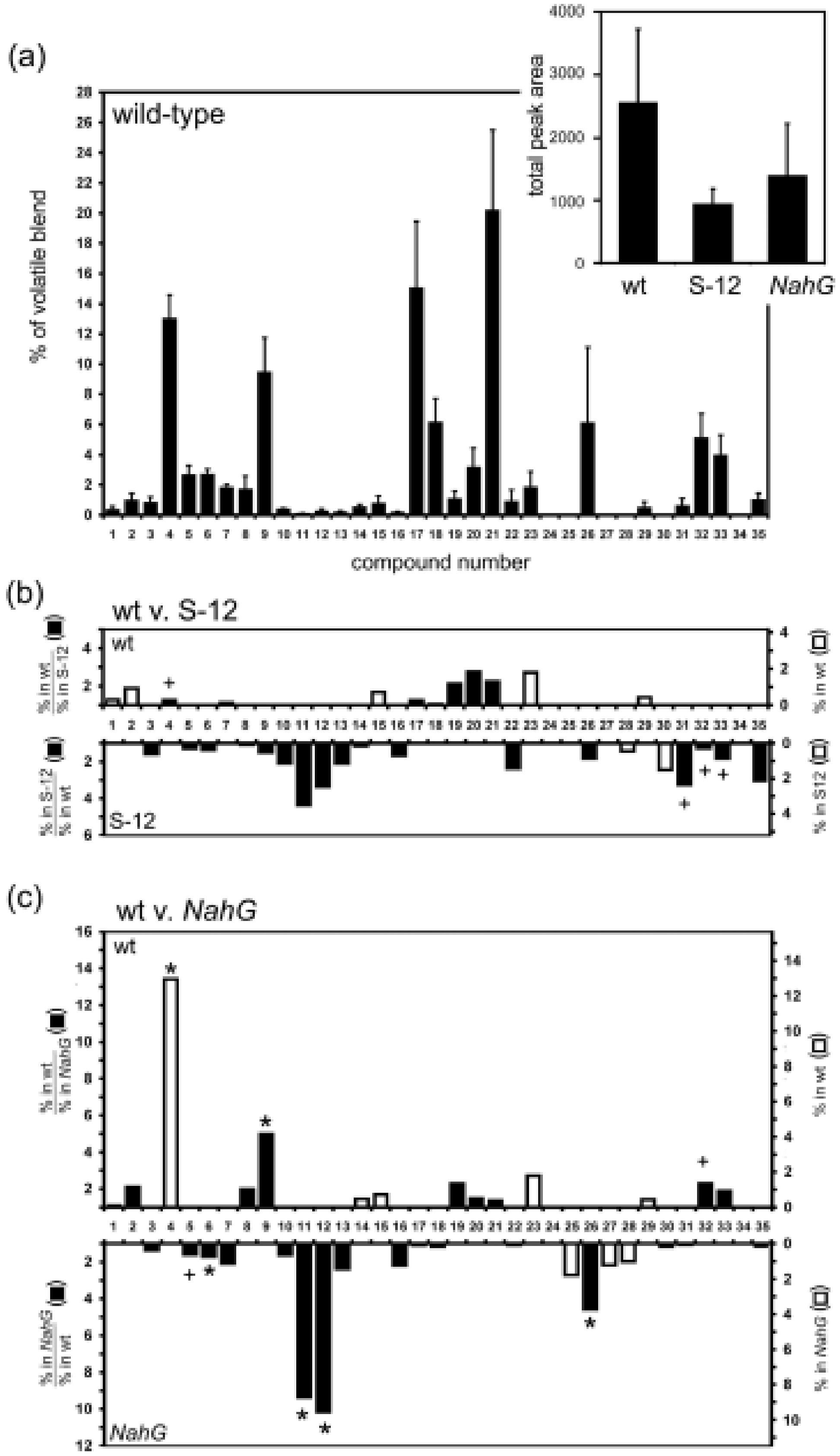
3. Results

3.1 Volatiles from P. rapae- infested wild-type Col0 and transgenic S-12, and NahG plants

After 24 h of feeding by *P. rapae*, wild-type *Arabidopsis* plants emitted a blend of volatiles consisting of green leaf volatiles (compound nrs 1-3), MeSA (4), terpenoids (5-9), dimethyl trisulphide (10), nitriles (11-15), methyl isothiocyanate (16), two compounds tentatively identified as cycloheptadienes based on mass spectra (17-18), alcohols (19-23), ketones (26, 29), aldehydes (31-33) and 1-nonene (35) (Figure 1a; see also Chapter 3).

Pieris rapae-infested S-12 plants, that do not show wound-induced accumulation of JA, produced on average 3 times lower amounts of volatiles than wild-type plants (Mann-Whitney U test, $P < 0.05$; Figure 1a insert). In contrast, differences in the relative amounts of the volatiles present in the blends of *P. rapae*-infested wild-type and S-12 plants are small (Figure 1b). None of the compounds showed a significant difference; four compounds were present in consistently higher or lower amounts in all three replicate pairs: MeSA (4) was present in slightly higher amounts (1.03-2 times higher) in the blend of *P. rapae*-infested wild-type plants, whereas octanal (31), nonanal (32) and decanal (33) were present in higher amounts in *P. rapae*-infested S-12 plants (octanal was not detected in two samples of wild-type plants, in the third sample 3 times lower compared to S-12, nonanal amounts were 1.1-2 times higher in S-12, and decanal amounts were1.4-4 times higher in S-12).

Pieris rapae-infested NahG plants, that are unable to accumulate SA (Delaney et al., 1994), produced lower amounts of volatiles than wild-type plants (1.2-3 times less; Figure 1a insert), but this was not statistically significant. If we compare the relative amounts of the volatiles present in the blends of *P. rapae*-infested wild-type and NahG plants, there are significant differences (Figure 1c).


(a)
wild-type
% of volatile blend
compound number
total peak area
wt
S-12
NahG
(b)
wt v. S-12
wt
S-12
% in wt / % in S-12
% in S-12 / % in wt
% in wt
% in S12
(c)
wt v. *NahG*
wt
NahG
% in wt / % in *NahG*
% in *NahG* / % in wt
% in wt
% in *NahG*

- Two compounds were emitted in relatively higher amounts from *P. rapae* infested wild-type plants (Mann-Whitney U test, $P < 0.05$); these are MeSA (nr 4; 10-15% in the wild-type samples, not detected in the NahG samples) and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT; nr 9; 7-14% in the wild-type samples, not detected in one sample and 3% in the other two samples of NahG).
- Four compounds were emitted in relatively higher amounts from *P. rapae*-infested NahG plants (Mann-Whitney U test, $P < 0.05$); these are limonene (nr 6; 4-6% in the NahG samples, 2-3% in the wild-type samples); 2-methylbutanenitrile (nr. 11; 0.3-0.7% in the NahG samples, not detected in two samples and 0.2% in the third sample of the wild-type plants); 3-methylbutanenitrile (12; 2-3% in the NahG samples, not detected in two samples and 0.6% in the third sample of wild-type plants) and 3-pentanone (26; 20-40% in the NahG samples, 0.7-16% in the wild-type samples).

Fi*h*u*s*e 1. Dpn ppt jujpo pgu f wprhujrn crhoe f n juf e cy *P. rapae*.jogf t uf e x jrn.uypf - T.23- boe *NahG Arabidopsis* prhout . Dpn ppvoe ost ;
)2))*Z*).3.i f yf o.2.prn)3) 2.i f ybopm)3) i f ybobm)4) n f ui ymt bjdymuf -)6) n ysdf of -
)6) rjm pof of -)8) rpohjgprhof -)9))*E,E*).α.gbsof t f of -
)9))3*E,7E*).4-9-23.usjn f ui ym2-3-8-22.usjef dbuf usbf of)TMTT)-)21) ejn f ui ymsjt vrgjef -
)22) 3.n f ui ymvubof ojusjm-)23) 3.n f ui ymvubof ojusjm-)23) 4.n f ui ympf oubof ojusjm-
)24) 6.)n f ui ymi jp).pf oubof ojusjm-)26) 6-8.eju jppdubof ojusjm-)26) n f ui ymjt pu jpdybobuf -
)28) 6.[)*Z*).2.cvuf oyrh2-4.dydmpi f pube jf of)uf oubujwf jef oujgdbujpo)-
)29) 6.cvuym2-4.dydmpi f pube jf of)uf oubujwf jef oujgdbujpo)-)29) 2.pf oubopm
)31) 3.pf oubopm)32) 2.pf ouf o.3.pm)33) 3.f ui ym2.i f ybopm)33) 2.opobopm
)34) 2.epef dbopm)36) 2.uf usbef dbopm)36) 3.pf oubopof -)38) 2.pf ouf o.3.pof -
)39) 3.n f ui ym3.pf oubopof -)39) 3.pdubopof -)31) i f pubobm)32) pdubobm)33) opobobm
)33) ef dbobm)34) 2.pduf of -)36) 2.opof of .

)b) Vprhujrnt efuf duf e jo ui f crhoe bt pf sdf oubhf pg ui f upubmpf bl bsf b pg wprhujrnt f n juf e cy *P. rapae*.jogf t uf e x jrn.uypf *Arabidopsis*. Ti f bwf sbhf boe tuboebse f ssps pg ui sf f sf prjdbuf t jt ti px o. Jotf su Tpubmbn pvou pg wprhujrnt f n juf e cy *P. rapae*.jogf t uf e x jrn.uypf - T.23 boe *NahG* prhout . Ti f bwf sbhf boe tuboebse ef wjbujpo pg ui sf f sf prjdbuf t jt ti px o.
)c) Dpn pbsjt po pg ui f wprhujrn crhoet f n juf e cy *P. rapae*.jogf t uf e x jrn.uypf)x u) boe *P. rapae*.jogf t uf e T.23 prhout x jui ui f joejwjevbmdpn ppvoet f ypsf t t f e bt pf sdf oubhf pg ui f upubmpf bl bsf b. Ti f di bsut i px t pf s dpn ppvoe ui f bwf sbhf pf sdf oubhf jo ui f x jrn.uypf crhoe ejwjef e cy ui f bwf sbhf pf sdf oubhf jo ui f T.23 crhoe)crhdl cbst- rhgu y.byjt). Jg ui jt wbmvf x bt mpx f s ui bo 2)x i jdi n f bot b i jhi f s pf sdf oubhf jo T.23 prhout ui bo jo x jrn. uypf prhout)- ui f jowf st f x bt ubl f o)cpupn pbsu pg di bsu). Jg b dpn ppvoe x bt gpvoe pomy jo x jrn.uypf)upp pbsu pg di bsu) ps T.23 prhout)cpupn pbsu pg di bsu) ui f bwf sbhf pf sdf oubhf jo ui jt crhoe jt ti px o)x i juf cbst - sjhi u y.byjt). Dpn ppvoet n bsl f e x jui bo btuf sjt l joejdbuf b tjhojgjdbou ejggf sf odf ; * P<1.16)Mboo.Wi juof y V uf t u)- dpn ppvoet n bsl f e x jui b , joejdbuf ui bujo bmui sf f pbjsf e sf prjdbuf t ui f psppsujpo pg ui jt dpn ppvoe x bt i jhi f s jo ui f crhoe pg T.23 prhout ui bo jo ui f crhoe pg ui f x jrn.uypf prhout ps wjdf wf st b.
)d) A t jn jrhs dpn pbsjt po bt jo)c) cvuopx x jui ui f wprhujrn crhoet f n juf e cy *P. rapae*. jogf t uf e x jrn.uypf)x u) boe *P. rapae*.jogf t uf e *NahG* prhout . Ti f di bsu t i px t pf s dpn ppvoe ui f bwf sbhf pf sdf oubhf jo ui f x jrn.uypf crhoe ejwjef e cy ui f bwf sbhf pf sdf oubhf jo ui f *NahG* crhoe)crhdl cbst - rhgu y.byjt). Jg ui jt wbmvf x bt mpx f s ui bo 2)x i jdi n f bot b i jhi f s pf sdf oubhf jo *NahG* prhout dpn pbsf e up x jrn.uypf prhout)- ui f jowf st f x bt ubl f o)cpupn pbsupg di bsu). Jg b dpn ppvoe x bt gpvoe pomy jo x jrn.uypf)upp pbsupg di bsu) ps *NahG* prhout)cpupn pbsupg di bsu) ui f bwf sbhf pf sdf oubhf jo ui jt crhoe jt ti px o)x i juf cbst - sjhi u y.byjt). Fps ui f f yprhobujpo pg bo btuf sjt l ps , t f f ui f rhhf oe pg gjhvsf 2C x jui *NahG* prhout jotuf be pg T.23 prhout .

Additionally, two compounds were present in consistently higher or lower amounts in all three replicate pairs:

- Myrcene (5) made up a larger part of the NahG sample compared to the corresponding wild-type sample in all three replicates (3-5% in the NahG samples, 1.3-2 times lower in the wild-type samples)
- Nonanal (32) made up a larger part of the wild-type sample compared to the corresponding NahG sample in all three replicates (2-7% of the wild-type samples, not detected in one sample and 1.2-1.3 times lower in the other two samples of the NahG plants).

3.1 Volatiles from jasmonic acid-treated plants

Spraying a 1.0 mM JA solution on wild-type Arabidopsis induced volatile production. Twenty-four hours after spraying, JA-treated plants emitted green leaf volatiles (compound nrs 1-3), methyl salicylate (4), terpenoids (5-9), two compounds tentatively identified as cycloheptadienes (17-18), alcohols (21-25), ketones (26-27), 3-octanone (29), aldehydes (32-33), and alkenes (34-35) (Figure 2a).

Compared to control plants, JA-treated plants emitted on average a 1.5 times higher total amount of volatiles (Figure 2a insert; Mann-Whitney U test, $P < 0.05$). If we compare the relative amounts of the volatiles present in the blends of JA-treated and control wild-type Arabidopsis, there are some striking differences (Figure 2b). The most remarkable difference between JA-treated and control plants is the induced emission of myrcene (5). It made up 1-6% of the volatile blend of JA-treated plants, but was hardly detected in the blend of control plants (Mann-Whitney U test, $P < 0.05$). Besides this, two compounds were present in consistently higher amounts in the blend of JA-treated plants in all three replicate pairs. These are MeSA (nr 4; 2-9% in the JA samples, 2-8 times lower in the control samples) and TMTT (nr 9; 0.4-26% in the JA samples, not detected in one sample and 8 times lower in the other two samples of control plants). A fourth compound that was on average present in higher amounts in the blend of JA-treated plants was (*E,E*)-α-farnesene (nr 8; not detected in one sample but 6-8% of the other two samples of the JA-treated plants, not detected in any of the three samples of control plants).

Three compounds were emitted in relatively higher amounts by control plants (Mann-Whitney U, $P < 0.05$). These are longifolene (nr 7; 9-14% in the control samples, 3-7% in the JA samples); 6-butyl-1,4-cycloheptadiene (nr 18; 10-17% in the control samples, 5-6% in the JA samples) and nonanal (nr 32; 6-8% in the control samples, 2-3% in the JA samples). Two other compounds were present in consistently higher amounts in the blend of control plants in all three replicate pairs: 6-[(Z)-1-butenyl]-1,4-cycloheptadiene (nr 17; 10-14% in the control samples, 1.3-1.4 times lower in the JA samples) and 1-tetradecanol (25; 7-13% in the control samples, 1.2-3 times lower in the JA samples).

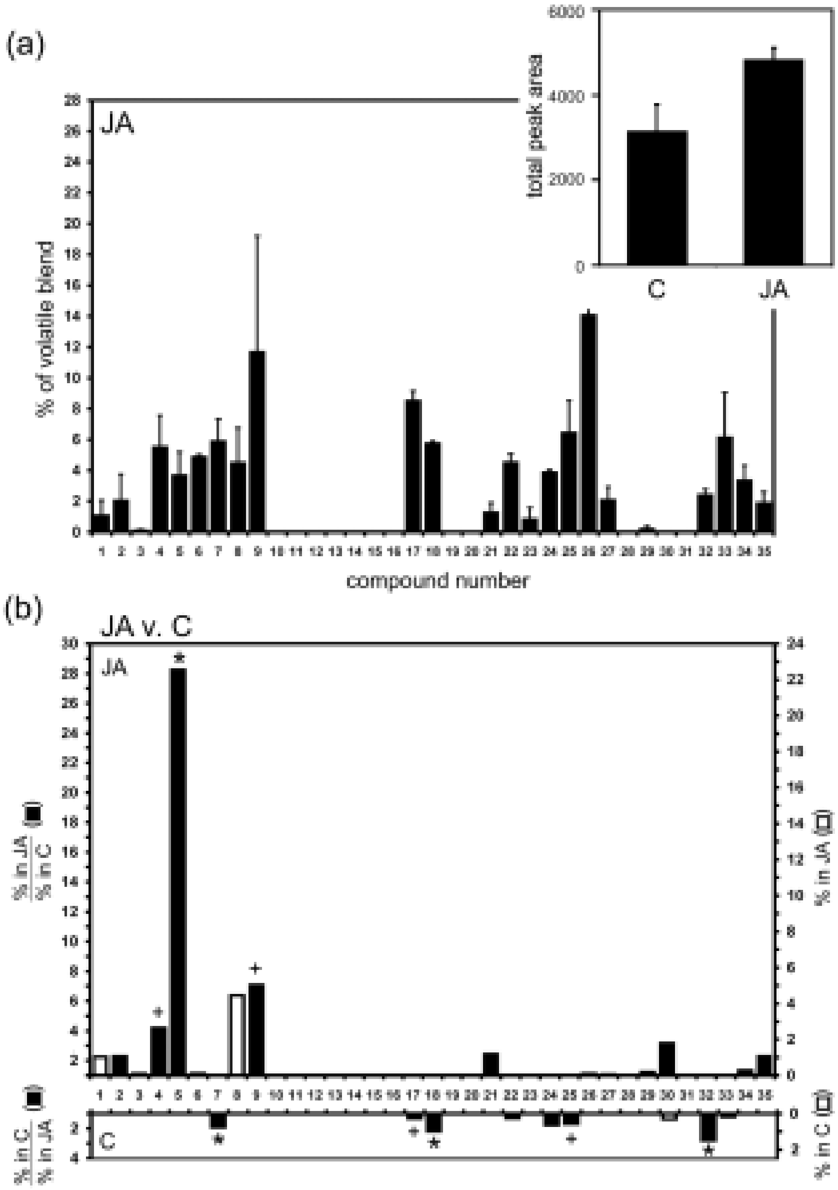


Figure 2. Composition of the volatile blend emitted by jasmonic-acid treated wild-type *Asac* **i***e opt* **i***t*. For compound numbers see legend of figure 1.

(a) Volatiles detected in the blend as percentage of the total peak area of volatiles emitted by *P.* ***s****apa***e**-infested wild-type *Asac* **i***e opt* **i***t*. The average and standard error of three replicates are shown. Insert: Total amount of volatiles emitted by control and JA treated wild-type plants. The average and standard deviation of three replicates are shown.

(b) Comparison of the volatile blends emitted by JA-treated and control (C) wild-type plants with the individual compounds expressed as percentage of the total peak area. The chart shows per compound the average percentage in the JA blend divided by the average percentage in the control blend (black bars, left y-axis). If this value was lower than 1 (which means a higher percentage in control plants compared to JA-treated plants), the inverse was taken (bottom part of chart). If a compound was found only in JA-treated (top part of chart) or control plants (bottom part of chart) the average percentage in this blend is shown (white bars, right y-axis). For the explanation of asterisk or + see the legend of figure 1b with *Nai G* plants instead of S-12 plants.

3.3 Gene-expression in P. rapae- infested wild-type, S-12, and NahG plants

We compared the expression of six genes between *P. rapae*-infested and uninfested wild-type, S-12, and NahG plants. Three genes are part of the octadecanoid pathway: *LOX2* encoding a wound-inducible lipoxygenase that is one of the first enzymes in the octadecanoid pathway (Bell et al., 1995); *AOS* encoding allene oxide synthase that directs the products of LOX in the OPDA and JA producing pathway (Laudert and Weiler, 1998); and *HPL*, encoding a hydroperoxide lyase that directs the products of LOX into a green leaf volatile producing pathway (Bate et al., 1998). Another gene, *PAL1*, encodes phenylalanine ammonia-lyase, that is, amongst others, involved in the production of SA and MeSA (Mauch-Mani and Slusarenko, 1996). The fifth gene *TPS10*, encodes a myrcene/(*E*)-β-ocimene synthase (Bohlmann et al., 2000). Expression of *UBQ*, a mixture of ubiquitin-encoding genes served as a constitutive control.

Only one of the six genes showed a distinct difference in expression between wild-type and S-12 plants (Figure 3). Expression of *LOX2* was 2-fold lower in undamaged S-12 plants compared to undamaged wild-type plants and 5-fold lower in *P. rapae*-infested S-12 plants than in *P. rapae*-infested wild-type plants. Whereas wild-type plants showed a 6-fold increase after 24 h of *P. rapae*-feeding, S-12 plants only showed a 2-fold increase. *Pieris rapae*-induced expression of *AOS*, *HPL*, *PAL1*, and *TPS10* was similar in wild-type and S-12 plants (Table 1). Expression of *UBQ* was equally expressed in undamaged and *P. rapae*-infested wild-type and S-12 plants.

When comparing NahG plants with wild-type plants, only expression of *HPL* showed a distinct difference (Figure 3). Expression of *HPL* was only 10-fold induced by *P. rapae*-feeding in NahG plants compared to a 53-fold induction in wild-type plants. This was due to both a higher expression in undamaged NahG plants (4-fold compared to undamaged wild-type plants) and a lower expression in *P. rapae*-infested NahG plants (1.6-fold lower compared to *P. rapae*-infested wild-type plants). *Pieris rapae*-induced expression of *LOX2*, *AOS*, *PAL1*, and *TPS10* was similar in wild-type and NahG plants (Table 1). Expression of *UBQ* was equally expressed in undamaged and *P. rapae*-infested wild-type and S-12 plants.

Tacle 1. Hfof fypsfttjpo jo voebnbhfe boe *P. rapae* jogftufe xjme.uypf- T.23 boe *NahG* pmbout sfmbujwf up fypsfttjpo jo voebnbhfe xjme.uypf pmbout.

hene	Wile -uype		T-12		NahG	
	voebnbhfe	*P. rapae*-jogftufe	voebnbhfe	*P. rapae*-jogftufe	voebnbhfe	*P. rapae*-jogftufe
LOX2	2	6.6	1.6	1.9	2.4	6.9
AOS	2	3.9	1.8	3.6	1.9	3.2
HPL	2	63	1.9	43	3.6	33.4
PAL1	2	4.8	2.2	4.6	2.2	4.6
TPS10	2	32	1.9	36	2.4	36
UBQ	2	1.8	2.6	2.1	2.4	2.1

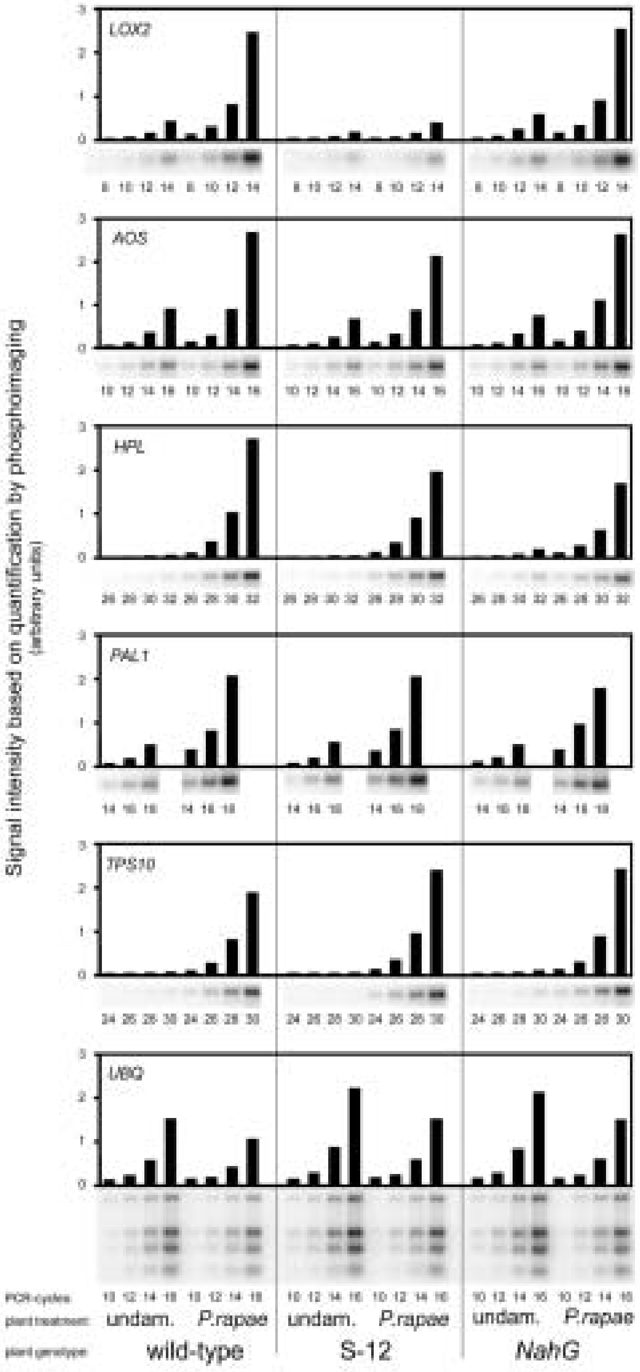


Figure **3**. ***G****ene-expression analysis of undamaged and *P*. *sapa*e-infested wild-type, S-12, and *Nai G Asac* **i***e opt* **i***t* plants. The figure shows the results of RT-PCR of 6 *Asac* **i***e opt* **i***t* genes: *LOX***2**, *AOT*, *HPL*, *PAL***1**, **T***PT***1***0*, and *UBQ*. Besides the images of the radioactive blots also the expression levels based on the number of counts from a phosphoimager are shown. The x-axis shows consecutive number of PCR-cycles on cD***N***A from the 6 different treatments. The y-axis is based on the number of counts by the phoshoimager. As the number of counts is dependent on the exposure time, which is different for the different genes, expression levels between different genes cannot be compared.

4. Discussion

4.1 Jasmonic acid induces the emission of terpenoids and methyl salicylate in *Ara*ahcnorhr

In the last decade, evidence has been accumulating that the octadecanoid pathway is involved in herbivore-induced emission of volatiles in several plant species (see Chapter 2 for a review). Indeed, spraying Arabidopsis with JA results in increased emission of volatiles, especially of MeSA, myrcene, (*E*,*E*)-α-farnesene and TMTT (Figure 2a and 2b). Emission of both MeSA and TMTT is also strongly induced in P. rapae-infested Arabidopsis, whereas myrcene emission is induced to a lesser extent by P. rapae-infestation and (*E*,*E*)-α-farnesene was previously not detected in the blend of P. rapae infested or uninfested Arabidopsis (Chapter 3). That JA application induces the emission of MeSA and TMTT in Arabidopsis is quite surprising. SA and JA act antagonistically in many plant responses (Baldwin et al., 1996; Niki et al., 1998; Felton et al., 1999) although synergism has also been reported (Xu, et al., 1994). Application of JA to Lima bean plants resulted in the emission of a volatile blend that was similar to the blend emitted by Lima bean plants infested with herbivorous spider mites, with the exception of a few compounds, of which MeSA and TMTT were most important! MeSA and TMTT emissions were highly induced by spider-mite feeding but hardly so by JA treatment (Dicke et al., 1999). Treatment of Lima bean with OPDA, an early intermediate of the octadecanoid pathway and a precursor for JA production, did induce TMTT emission (Koch et al., 1999). The authors concluded that with respect to terpenoids, in Lima bean JA (a late intermediate of the octadecanoid pathway) can trigger the biosynthesis of mono- and sesquiterpenes (such as myrcene and (*E*,*E*)-α-farenese), whereas OPDA can trigger the biosynthesis of diterpenoids (or diterpenoid derived compounds, such as TMTT). In Arabidopsis we see that JA induces the emission of myrcene and (*E*,*E*)-α-farnesene but also of MeSA and TMTT. However, the relative proportions of these compounds are somewhat different compared to P. rapae-infested Arabidopsis (Figures 1a and 2a; Chapter 3). This difference in proportions suggests that other signalling compounds are also involved in herbivory-induced volatile production in Arabidopsis.

4.2 Are LOX2-cosuppressed S-12 plants altered in volatile emission rather than volatile production?

As the octadecanoid pathway is clearly involved in herbivore-induced volatile emission, we studied the volatile emissions of P. rapae-infested wild-type and S-12 Arabidopsis. The S-12 plants are LOX2 cosuppressed and therefore do not show wound-induced accumulation of JA (Bell et al., 1995). Volatile analysis shows that after 24 h of P. rapae infestation, S-12 plants produce a significantly lower amount of volatiles than wild-type plants (Figure 1a insert). However, the ratios between different compounds in the volatile blend hardly differ between infested wild-type and S-12 plants (Figure 1b). This means that all compounds are emitted in lower *amounts by* S-*12 plants, including compounds that are emitted in equal amounts in undamaged and P. rapae infested wild-type plants. A possible explanation is that volatiles produced by* S-*12 plants are less easily emitted.* J*A has been reported to be involved in the development of stomata* (B*ourgouin and* H*orton, 1*992). I*ndeed, preliminary data show that the leaf surface of* S-*12 plants contains two-fold less stomata per surface unit compared to wild-type plants* (R.M.*P.* V*an* P*oecke, unpublished data*). *An additional factor that might influence volatile emission in* S-*12 plants is that these plants lack trichomes on the leaves, reducing leaf surface. Although both phenotypical differences can explain why* S-*12 plants emit lower amounts of volatiles, they do not explain why we do not see an effect on* J*A-inducible volatiles like myrcene,* (*E,E*)-α-*farnesene,* Me*SA and* TMTT. W*e would have expected that especially the emission of* Me*SA and* TMTT, *which are strongly induced by P. rapae feeding in wild-type plants* [40 *resp. 1*3-*fold compared to undamaged wild-type plants* (*Chapter* 3)], *would be much more reduced in* S-*12 plants than the 2 to* 3-*fold reduction that we see on average for all compounds.* H*owever, using the same* S-*12 Arabidopsis Pieterse et al.* (200*1*) *found that induced systemic resistance against pathogens, although dependent on* J*A-signalling, did not require an increase in* J*A production.* T*hey suggested that the* J*A-dependency was based on enhanced sensitivity to* J*A.* I*f this is also true for herbivory-induce volatile production, then both increased production of and increased sensitivity to* J*A can induce the production of volatiles.* O*ther explanations are that LOX2 expression is not rate limiting or the small induction of LOX2 observed in* S-*12 plants by P. rapae-infestation is enough to induce volatile production. All three hypotheses also explain the limited effect of LOX2 cosuppression on herbivory-induced expression of genes that are likely to be involved in volatile emissions* (F*igure* 3). B*esides the expected reduced induction of LOX2 in* S-*12 plants by P. rapae feeding, there are hardly any effects on HPL, AOS, PAL1 and TPS10 induction, even though AOS is known to be induced by* J*A in Arabidopsis and PAL genes are known to be induced by* J*A in other plant species* (*Creelman et al., 1*992; *Gundlach et al., 1*992; L*audert and* W*eiler, 1*998).

4.3 *Pieris rapae*-induced emission of MeSA and TMTT is dependent on salicylic acid

I*n constrast to the* S-*12 plants, NahG plants that do not accumulate* S*A emit a volatile blend that is significantly different from the blend of wild-type plants* (F*igure 2c*). T*his is mainly due to the reduced production of* Me*SA and* TMTT. W*hereas* Me*SA and* TMTT *constitute respectively 1*3% *and* 9% *of the volatile blend of P. rapae-infested wild-type plant,* Me*SA is not detected in the volatile blend of P. rapae-infested NahG plants and* TMTT *constitutes* 2% *of that blend. Although an absence of* Me*SA production by NahG plants was to be expected, the reduced production of* TMTT *demonstrates that* S*A is not only involved as a precursor of* Me*SA in herbivory-induced volatile production by Arabidopsis, but also as a signalling compound.*

4.4 Comparing the role of the octadecanoid and salicylic acid pathway in herbivore-induced volatile emissions between Lima bean and *Ara*alcnorlr

In contrast to the antagonistic effects of SA and JA in other defence responses, both SA and JA are involved in herbivory-induced volatile production by Arabidopsis. Similarly, Ozawa et al. (2000) suggested that both JA-related and SA-related signalling pathways are involved in herbivory-induced volatile production by Lima bean. Treatment of Lima bean plants with MeSA induced the production of the homoterpenes TMTT and to a lesser extend (*E*)-4,8-dimethyl-1,3,7-nonatriene. Treatment of Lima bean plants with JA followed by treatment with MeSA resulted in a volatile blend that showed higher similarity to the blend of spider-mite infested plants than treatment with JA alone, with one of the major differences being the induced production of TMTT after the combined JA/MeSA treatment. As OPDA also induces the production of TMTT in Lima bean (Koch et al., 1999) and SA is reported to promote OPDA but not JA production in Arabidopsis (Laudert and Weiler, 1998), SA might be involved as an inducer of OPDA.

A difference between Arabidopsis and Lima bean is that both caterpillar feeding and JA treatment induce MeSA and TMTT in Arabidopsis but not in Lima bean (Dicke et al., 1999; Ozawa et al., 2000; this paper). Indeed, the volatile blend of JA-treated Lima bean plants closely resembles the volatile blend of caterpillar-infested Lima bean plants. This suggests that in Lima bean, caterpillar feeding induces JA but not SA (and/or MeSA) levels, while spider-mite feeding induces both, with a subsequent inhibitory effect of SA on JA and a stimulatory effect of SA on OPDA (Figure 4a). Such a temporal separation of JA, SA and OPDA induction has been shown for the interaction between Lima bean and alamethicin, an ion channel-forming peptide mixture from the plant pathogen Trichoderma viride. Alamethicin treatment of Lima bean induced elevated JA levels in the first 90 min after treatment. After 90 min SA levels rose and JA levels dropped. The rise in SA levels resulted in a rise of OPDA levels (Engelberth et al., 2001).

In Arabidopsis, caterpillar feeding induces elevated JA levels (Stotz et al., 2002) and elevated JA levels induce MeSA (Figure 2b) and therefore most likely also SA levels. In contrast to the situation in Lima bean, elevated SA levels have on a minor effect on JA synthesis in Arabidopsis (Laudert and Weiler, 1998; Stotz et al., 2002), but similar to Lima bean, it does promote production of OPDA (Laudert and Weiler, 1998). The production of OPDA takes place in the chloroplast, and for subsequent conversion to JA it must be transported to the cytosol (Laudert and Weiler, 1998). The latter authors speculated that an increase of OPDA levels without a subsequent increase in JA levels upon SA treatment was due to an increased influx of substrate into the octadecanoid pathway in combination with an inhibitory effect of SA on OPDA transport from the chloroplast to the cytosol. Diterpenoid synthesis also takes place in the chloroplast (Lichtenthaler, 1999), so for the OPDA-induced production of diterpenoids no transport to the cytosol is needed (Koch et al., 1999). The inhibitory role of SA on OPDA transport to the cytosol is not complete as SA has only a minor effect on JA levels (Laudert and Weiler, 1998; Stotz et al., 2002). More likely, SA directs the increased influx of

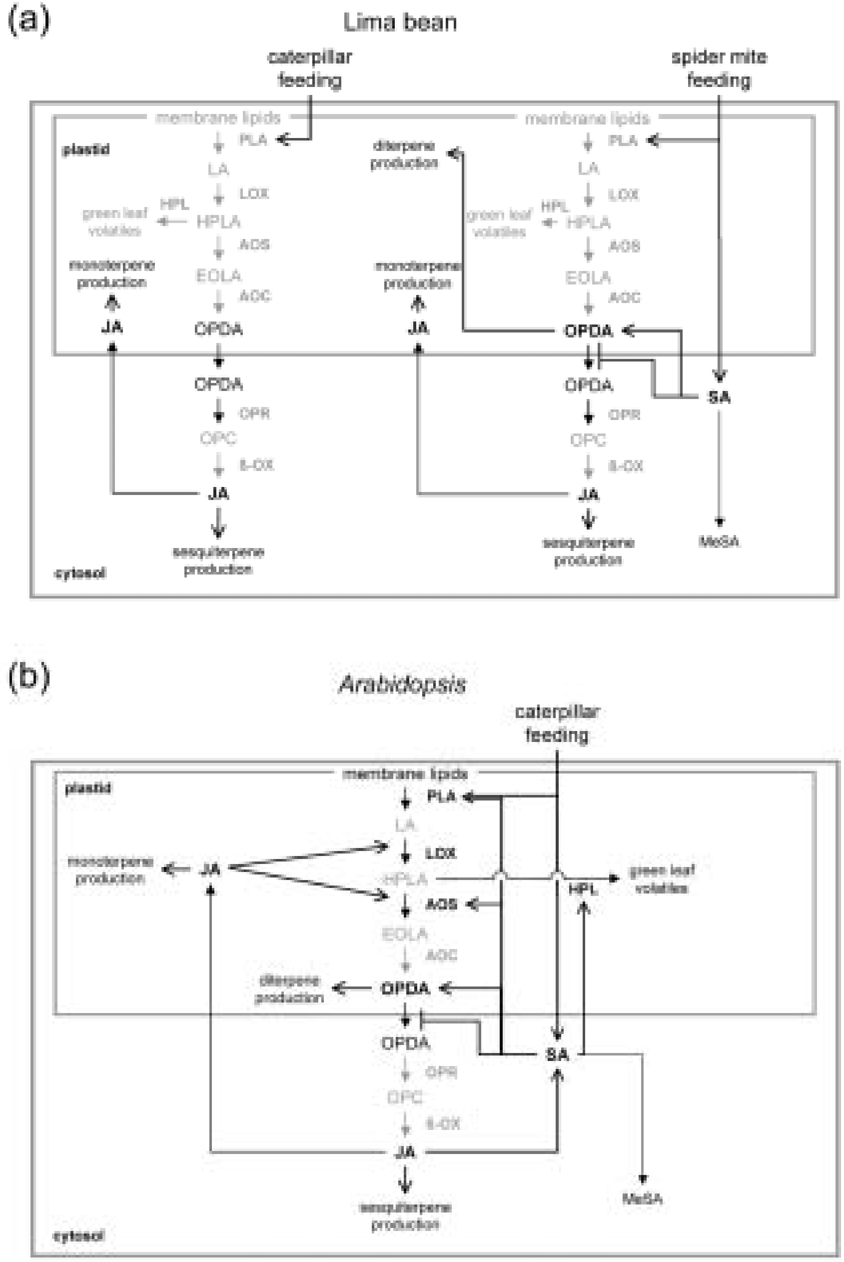


Figure 4. Mpeelt eetdsibioh ti e tihobl-tsboteudtipo pbtiwby io iesbivpsy-ioeudee vplbtile pspeudtipo.
(b) Sihobl-tsboteudtipo pbtiwbyt iovplvee io dbtespillbs boe tpiees-nite ioeudee vplbtile pspeudtipo io Linb bebo.
(b) Sihobl-tsboteudtipo pbtiwbyt iovplvee io dbtespillbs ioeudee vplbtile pspeudtipo io *Arabidopsis*.
Asspwt witi tplie bsspwiebet ioeidbte biptyotietid pbtiwbyt- pties bsspwt ioeidbte ioeudtipo ps ioi ibitipo. Eozynet ps pspeudtt io hsey bse opt neotipoee io tie text. PLA- piptpi plipbte A; LPX- lippxyheobte; APS- blleoe pxiee tyotiete; APC- blleoe pxiee dydlbte; PPR- PPDA seeudtbte; β-PX- β-pxiebtipo; HPL iyesppespxiee lybte; LA- α-liopleoid bdie; HPLA- 13(*S*)-iyesppespxyliopleoid bdie; EPLA- 12-13-eppxyliopleoid bdie; PPDA- 12-pxppiytpeieopid bdie; PPC- 3-pxp-2-(2(*Z*)-peoteoyl)-dydlppsppboe-1-pdtbopie bdie; JA- jbtnpoid bdie; SA- tblidylid bdie; MeSA- netiyl tblidylbte. Fps b seview po tie pdtbeedbopie pbtiwby tee Sdiblles (2001).

substrate into the octadecanoid pathway to OPDA production. As such, both SA (via OPDA) and JA can simultaneously induce volatile emissions (Figure 4*b*). The effect of SA on OPDA transport might be dose-dependent. This could explain why in Lima bean alamethicin induces only diterpenes and MeSA production whereas spider mites induce also the JA-dependent production of mono- and sesquiterpenes (Dicke *et al.*, 1999; Engelberth *et al.*, 2001). As a pathogen-derived elicitor, alamethicin possibly induces SA to a higher level compared to spider mite feeding, resulting in a complete block of OPDA transportation after alamethicin treatment and only a partial block after spider mite feeding. Besides the induction of MeSA through JA, caterpillar feeding probably induces MeSA in another way (e.g. through induced *PAL1* activity), as the amount of MeSA emitted by *P. rapae*-infested *Arabidopsis* is larger compared to JA-treated *Arabidopsis*.

4.5 The reduced production of TMTT by NahG plants is not due to a reduced induction of AOS expression

Although TMTT production is reduced in NahG plants, this is not dependent on the induction of *AOS* by SA. Induced expression of *AOS* by *P. rapae* in NahG plants was not different from wild-type plants (Figure 3). This suggests that in NahG plants, other factors involved in OPDA production are rate limiting, such as SA-dependent phospholipase A (PLA) activity, as suggested by Laudert *et al.* (1998; see also Figure 4*b*). The induction of other genes, such as *LOX2*, *PAL1* and *TPS10* was also not affected in NahG plants, except for the expression of *HPL*. The induction by *P. rapae*-feeding of *HPL* was reduced in NahG plants, indicating that SA might be involved in *P. rapae*- induced emission of green leaf volatiles. However, green leaf volatiles were only detected in trace amounts in the blend of both *P. rapae* infested wild-type and NahG plants, so we cannot draw a conclusion on the effect of reduced *HPL* induction in NahG plants on green leaf volatile production.

4.6 Differences in volatile blends correlate with differences in parasitoid attraction

In this study we demonstrated an effect of JA treatment, *LOX2* cosuppression, and lack of SA on volatile emissions by *Arabidopsis*. In a previous study (Chapter 4), we reported the effects of JA treatment, *LOX2* cosuppression, and lack of SA on parasitoid attraction by *Arabidopsis*. Comparing these studies results in the following: Treatment of plants with JA induced volatiles that are also induced by *P. rapae* feeding, except for the production of nitriles, which were not produced by JA-treated plants. The ratio's in which these volatiles are present in the blend differs between JA-treated and *P. rapae*-infested plants especially for MeSA and terpenoids. Females of the parasitoid wasp *Cotesia rubecula*, that is a specific natural enemy of *P. rapae*, were more attracted to JA-treated and *P. rapae*-infested plants compared to undamaged plants and they could also discriminate between JA-treated and *P. rapae*-infested plants, preferring the latter. This indicates that nitriles, MeSA, and terpenoids like TMTT are important volatile cues for *C. rubecula* females in the search for their host. Indeed, *C. rubecula* was less attracted to P. rapae-infested NahG plants than to P. rapae-infested wild-type plants, and the major difference in the volatile blends was that NahG plants produced much less MeSA and TMTT. That infested NahG plants are more attractive than undamaged NahG plants demonstrates that MeSA and TMTT are not the only attractive compounds in the blend. SA treatment of wild type plants did not result in an increased attraction of parasitoid wasps (Chapter 4). According to the model shown in Figure 4b, we would expect that SA-treated plants emit more MeSA and TMTT and therefore attract more parasitoids. This implies that although SA induces OPDA production, an additional signal (JA?) is needed before elevated OPDA levels induce TMTT and possibly also for the production of MeSA from SA, or that, although MeSA and TMTT are important volatile cues, emission of these volatiles without the volatiles induced by JA does not result in attraction of C. rubecula.

That P. rapae-infested S-12 plants attract fewer wasps than infested wild-type plants is not surprising, as S-12 plants were shown to emit much lower quantities of volatiles irrespective of their identity.

4.7 Conclusions

Recently, it has been argued that in the field of chemical ecology of insect-plant interactions, molecular approaches have a great potential to elucidate the role of secondary plant metabolites (Mitchell-Olds et al., 1998; Roda and Baldwin 2002; Dicke et al., 2002). The number of publications on direct defences of plants against herbivorous insects using molecular approaches is rapidly increasing, but in the field of indirect defences the use of this approach has been lagging (Kessler and Baldwin, 2002; Chapter 2). With different treatments and different Arabidopsis genotypes as input and behavioural (Chapter 4), chemical and gene-expression analysis (this Chapter) as output, we have provided a detailed improvement of the understanding of the signalling role of both JA and SA in indirect plant defences of Arabidopsis. Furthermore, combined with other publications, these studies indicate a possible role for OPDA as a signalling compound and of MeSA and TMTT as parasitoid attractants. Future research should demonstrate whether such important roles for OPDA, MeSA and TMTT can indeed be found.

Acknowledgments

We thank Casper Pillen and Aart van Ommeren for rearing of Arabidopsis plants, Leo Koopman, Frans van Aggelen and Andre Gidding for the insect rearing, Jörg Bohlmann and Jonathan Gershenzon for the TPS10 plasmids, Steven Rothstein, Jocelyne Le Tarte and Richard Stahl for providing the LOX2, AOS and HPL plasmids, and Tom Ruttink for the UBQ plasmids and the use of STORM. M.D. was partly funded by the Uyttenboogaart-Eliasen Foundation, Amsterdam.

References

Baldwin, I.T., Schmelz, E.A., Zhang, Z.P. (1996) Effects of octadecanoid metabolites and inhibitors on induced nicotine accumulation in Nicotiana sylvestris. J. Chem. Ecol., 22, 61-74.

Bate, N.J., Sivasankar, S., Moxon, C., Riley, J.M.C., Thompson, J.E., Rothstein, S.J. (1998) Molecular characterization of an Arabidopsis gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible. Plant Physiol., 117, 1393-1400.

Bell, E., Creelman, R.A., Mullet, J.E. (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc. Natl. Acad. Sci. USA, 92, 8675-8679.

Bohlmann, J., Martin, D., Oldham, N.J., Gershenzon, J. (2000) Terpenoid secondary metabolism in Arabidopsis thaliana: cDNA cloning, characterization, and functional expression of a myrcene/(E)-beta-ocimene synthase. Arch. Biochem. Biophys., 375, 261-269.

Bourgouin, N.C., Horton, R.F. (1992) Heterophylly in amphibious Ranunculi: the effects of abscisic acid and methyl jasmonate. In Current Plant Science and Biotechnology in Agriculture Vol 13: Progress in plant growth regulation: proceedings of the 14th international conference on plant growth substances held in Amsterdam on 21-26 July 1991. (Karssen, C.M., Loon, L.C. van, Vreugdenhil, D.) Dordrecht, the Netherlands: Kluwer Academic Publishers, pp. 386-392.

Creelman, R.A., Tierney, M.L., Mullet, J.E. (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl. Acad. Sci. USA, 89, 4938-4941.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut Rella, M., Kessmann, H., Ward, E., Ryals, J. (1994) A central role of salicylic acid in plant disease resistance. Science, 266, 1247-1250.

Dicke, M. (1999a) Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? Entomol. Exp. Appl., 91, 131-142.

Dicke, M. (1999b) Evolution of induced indirect defense of plants. In The Ecology And Evolution Of Inducible Defenses. (Tollrian, R., Harvell, C.J., eds).Princeton, New Jersey: Princeton University Press, pp 62-88.

Dicke, M., Gols, R., Ludeking, D., Posthumus, M.A. (1999) Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. J. Chem. Ecol., 25, 1907-1922.

Dicke, M., Van Beek, T.A., Posthumus, M A., Ben Dom, N., Van Bokhoven, H., de Groot, A.E. (1990) Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. J. Chem. Ecol., 16, 381-396.

Dicke, M., Van Poecke, R.M.P. and de Boer, J.G. (2002) Inducible indirect defence of plants: from subcellular mechanisms to ecological functions. Basic Appl. Ecol., in press.

Engelberth, J., Koch, T., Schuler, G., Bachmann, N., Rechtenbach, J., Boland, W. (2001) Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean. Plant Physiol., 125, 369-377.

Felton, G.W., Korth, K.L., Bi, J.L., Wesley, S.V., Huhman, D.V., Mathews, M.C., Murphy, J.B., Lamb, C., Dixon, R.A. (1999) Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. Curr. Biol., 9, 317-320.

Fritzsche-Hoballah, M.E., Turlings, T.C.J. (2001) Experimental evidence that plants under caterpillar attack may benefit from attracting parasitoids. Evol. Ecol. Res., 3, 553-565.

Geervliet, J.B.F., Posthumus, M.A., Vet, L.E.M., Dicke, M. (1997) Comparative analysis of headspace volatiles from different caterpillar-infested or uninfested food plants of Pieris species. J. Chem. Ecol., 23, 2935-2954.

Gundlach, H., Muller, M.J., Kutchan, T.M., Zenk, M.H. (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. USA, 89, 2389-2393.

Karban, R., Baldwin, I.T. (1997) Induced Responses to Herbivory. Chicago, USA:. The University of Chicago Press Ltd.

Kessler, A., Baldwin, I.T. (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science, 291, 2141-2144.

Kessler, A., Baldwin, I.T. (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annu. Rev. Plant Biol., 53, 299-328.

Koch, T., Krumm, T., Jung, V., Engelberth, J., Boland, W. (1999) Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. Plant Physiol., 121, 153-162.

Laudert, D., Weiler, E.W. (1998) Allene oxide synthase: a major control point in Arabidopsis thaliana octadecanoid signalling. Plant J., 15, 675-684.

Lichtenthaler, H.K. (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol., 50, 47-65.

Mauch-Mani, B., Slusarenko, A. J. (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of arabidopsis to Peronospora parasitica. Plant Cell, 8, 203-212

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., Browse, J. (1997) Jasmonate is essential for insect defense Arabidopsis. Proc. Natl. Acad. Sci. USA, 94, 5473-5477.

Mattiacci, L., Dicke, M., Posthumus, M.A. (1995) β-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. Proc. Natl. Acad. Sci. USA, 92, 2036-2040.

Mitchell-Olds, T., Gershenzon, J., Baldwin, I.T., Boland, W. (1998) research focus - Chemical ecology in the molecular era. Trends in Plant Science, 3, 362-365.

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N., Ohashi, Y. (1998) Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. Plant Cell Physiol., 39, 500-507.

Ozawa, R., Arimura, G., Takabayashi, J., Shimoda, T., Nishioka, T. (2000) Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. Plant Cell Physiol., 41, 391-398.

Pare, P. W., Tumlinson, J.H. (1997) De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol., 114, 1161-1167.

Piel, J., Atzorn, R., Gabler, R., Kuhnemann, F., Boland, W. (1997) Cellulysin from the plant parasitic fungus Trichoderma viride elicits volatile biosynthesis in higher plants via the octadecanoid signalling cascade. FEBS Lett., 416, 143-148.

Pieterse, C.M.J., van Loon, C.L. (1999) Salicylic acid-independent plant defence pathways. Trends Plant Sci., 4, 52-58.

Pieterse, C.M.J., Van Pelt, J.A., Van Wees, S.C.M., Ton, J., Léon-Kloosterziel, K.M., Keurentjes, J.J.B., Verhagen, B.W.M., Knoester, M., Van der Sluis, I., Bakker, P.A.H.M, Van Loon, L.C. (2001) Rhizobacteria-mediated induced systemic resistance: triggering, signalling and expression. Eur. J. Plant Pathol., 107, 51-61.

Reymond, P., Weber, H., Damond, M., Farmer, E.E. (2000) Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell, 12, 707-719.

Roda, A., Baldwin, I.T. (2002) Molecular technology reveals how the induced direct defenses of plants work. Basic Appl. Ecol., in press.

Schaller, F. (2001) Enzymes of the biosynthesis of octadecanoid-derived signalling molecules. J. Exp. Bot., 52, 11-23.

Stotz, H.U., Koch, T., Biedermann, A., Weniger, K., Boland, W., Mitchell-Olds, T. (2002) Evidence for regulation of resistance in Arabidopsis to Egyptian cotton worm by salicylic and jasmonic acid signalling pathways. Planta, 214, 648-652.

Turlings, T.C.J., Loughrin, J.H., McCall P.J., Röse U.S.R., Lewis, W.J., Tumlinson, J.H. (1995). How caterpillar-damaged plants protect themselves by attracting parasitic wasps. Proc Natl Acad Sci USA 92, 4169-4174.

Van Loon, J.J.A., de Boer, J.G., Dicke, M. (2000) Parasitoid-plant mutualism: parasitoid attack of herbivore increases plant reproduction. Entomol. Exp. Appl., 97, 219-227.

Xu, Y., Chang, P.F.L., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M., Bressan, R.A. (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell, 6, 1077-1085.

chapter

6

Signal transduction downstream of salicylic and jasmonic acid in herbivory-induced parasitoid attraction by *Arabidopsis*.

Remco M. P. van Poecke and Marcel Dicke

"All things come into being through opposition, and all are in flux, like a river."

Heraclitus

Chapter 6

SIGNAL TRANSDUCTION DOWNSTREAM OF SALICYLIC AND JASMONIC ACID IN HERBIVORY-INDUCED PARASITOID ATTRACTION BY *Arabidopsis*.

Abstract

Plants can defend themselves indirectly against herbivores by attracting natural enemies of these herbivores, either predators or parasitoids. They can attract these carnivores by emitting a volatile blend upon herbivory. Besides the mechanical wounding inflicted by herbivores, herbivore-derived elicitors are involved in the induced production of volatiles and increased attraction of carnivores. The signal transduction pathway in plants from herbivory to induced volatile production depends on jasmonic acid (JA) and salicylic acid (SA). Recent work using *Arabidopsis thaliana* confirmed this. Although JA and SA are involved in herbivory-induced volatile production, the pathways downstream of JA and SA are unknown. Use of *Arabidopsis* provides a unique possibility of studying signal transduction pathways by use of signalling mutants. However, the use of these signalling mutants has not been exploited in studies on indirect plant defence. Here we demonstrate that herbivore-derived elicitors are involved in herbivory-induced attraction of the parasitoid *Cotesia rubecula*. Moreover, using *jar1-1* and *npr1-1* mutants we studied the role of *JAR1* and *NPR1* in signal transduction downstream of JA and SA in the induction of carnivore attractants. Both *jar1-1* and *npr1-1* are known to be involved in signalling downstream of JA in the defence against pathogens such as induced systemic resistance (ISR). The *npr1-1* is also involved in signalling downstream of SA in the defence against pathogens, such as systemic acquired resistance (SAR). We show that *jar1-1* and *npr1-1* are not affected in the induced attraction of *C. rubecula* by *Arabidopsis* infested by *Pieris rapae* caterpillars. These results shed new light on cross-talk between signalling pathways involved in defence against herbivores and pathogens.

---

1. Introduction

To defend themselves against herbivorous insects, plants employ both direct and indirect defences (Karban and Baldwin, 1997; Dicke, 1999). Direct defence mechanisms directly affect the herbivore, e.g. by a negative impact on the physiology of the herbivore through toxic or anti-nutritional compounds, or by interfering with the behaviour of the herbivore through repellents or deterrents (Karban and Baldwin, 1997). Indirect defence mechanisms promote the effectiveness of natural enemies of the herbivore, i.e. predators or parasitoids. One way of promoting the effectiveness of natural enemies is by producing volatiles in response to herbivory that can attract predators or parasitoids. This kind of tritrophic interaction has been shown for many plant species, belonging to at least twelve plant families, in combination with a diverse range of herbivore and natural enemy species (Dicke, 1999).

Many of the defence mechanisms are induced upon infestation with pathogens or herbivores, including the emission of volatiles. Such inducibility requires signal processing in the plant and many different signalling compounds have been identified. Plant hormones like salicylic acid (SA), ethylene (Eth), and jasmonic acid (JA) and 12-oxo-phytodienoic acid (OPDA) are especially important in induced plant defences (Karban & Baldwin 1997; Glazebrook, 2001; Stintzi *et al.*, 2001; Chapter 2). The signal transduction pathways involved in the induced responses of a plant to wounding, herbivory and pathogen infection often overlap. Jasmonic acid, for example, is involved in wound-induced responses, inducible defences against insects, including herbivory-induced volatile production, and inducible defences against pathogens (Berger, 2002; Pieterse *et al*, 2001b; Chapter 2). Likewise, salicylic acid is involved in herbivory-induced volatiles production and inducible defences against pathogens (Ozawa *et al.*, 2000; Dong, 2001; Chapters 4 and 5).

Although the signal-transduction pathways often overlap, the induced responses of a plant to wounding, herbivory and pathogen infection are often quite different. For example, in *Arabidopsis* wounding results in the induced expression of many genes that are also induced by caterpillar feeding. Moreover, wounding also induced the induction of many water-stress-inducible genes that are not induced by caterpillar feeding (Reymond *et al.*, 2000). The differences between caterpillar feeding and wounding can be explained by the presence of elicitors in the regurgitant of the caterpillar. For example, caterpillar-feeding or treatment of artificially damaged plants with caterpillar regurgitant induces higher levels of JA in tobacco or phenolics in birch and causes a more rapid induction of *HMG* (3-hydroxy-3-methylglutaryl-coenzyme A reductase) and *PIN2* (proteinase inhibitor 2) gene-expression in potato compared to mechanically damaged plants (Hartley and Lawton, 1991; Korth and Dixon, 1997; McCloud and Baldwin, 1997). Similar results were obtained with respect to herbivory-induced volatile production. Although artificial damage often results in increased volatile production and may lead to parasitoid attraction, herbivory or treatment with caterpillar regurgitant results in the emission of a quantitatively and/or qualitatively different volatile blend that is emitted for a longer period after termination of the treatment compared to wounding. These herbivore infested or regurgitant treated plants are more attractive to parasitoids than undamaged or mechanically damaged plants (Turlings *et al.*, 1990; Steinberg *et al.*, 1993; Mattiacci *et al.*, 1994; Takabayashi *et al.*, 1995). Several elicitors have been isolated from caterpillar regurgitant: β-glucosidase from *Pieris brassicae* regurgitant and fatty acid-amino acid conjugates from *Spodoptera exigua* (volicitin), *Manduca sexta* and *M. quinquemaculata*. Treatment of plants with regurgitant-derived elicitors results in an emission of volatiles and to an increase in JA levels that is comparable to plants treated with regurgitant (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Halitschke *et al.*, 2001). Besides eliciting compounds, also suppressing compounds have been found in caterpillar regurgitant. Glucose oxidase in the saliva and regurgitant of the herbivore *Helicoverpa zea* suppressed a direct defence mechanism of tobacco (Musser *et al.*, 2002).

Responses to herbivory and pathogens defence can also be very different, sometimes resulting in negatively correlated defences. Tobacco plants infested with tobacco mosaic virus (TMV), resulting in increased SA levels, were more susceptible to *Manduca sexta* caterpillars (Preston *et al.*, 1999) and transgenic tobacco plants with reduced SA levels were more susceptible to TMV but showed increase resistance against *Heliothis virescens* caterpillars (Felton *et al.*, 1999). In these cases, signal transduction pathways might not be overlapping but antagonistic. Salicylic acid is known to act antagonistically on JA-inducible responses and vice versa (Niki *et al.*, 1998; Felton and Korth, 2000; Pieterse *et al.*, 2001a). Although from these results it might appear that pathogens induce SA-dependent responses and herbivorous insects induce JA-dependent responses that negatively interact, such a generalisation is an oversimplification. First of all, some pathogen-induced defences are JA-dependent and SA-independent (Penninckx *et al.*, 1998; Staswick *et al*, 1998; Thomma *et al.*, 1998; Vijayan *et al.*, 1998). Moreover, induced systemic resistance (ISR) against pathogens induced by non-pathogenic rhizobacteria is JA-dependent and SA-independent, but can act synergistically with systemic acquired resistance (SAR) against pathogens, that is induced by biotrophic pathogens and is SA-dependent and JA-independent (Pieterse *et al.*, 2001b). This demonstrates that JA and SA-dependent signalling pathways can act synergistically. Similarly, both JA and SA are involved in herbivory-induced volatile production (Ozawa *et al.*, 2000; Chapters 4 and 5). The relative importance of JA-dependent and SA-dependent signalling pathways might be dependent on herbivore-species, with chewing insects mainly inducing defences through the wound-inducible JA pathway, and phloem-feeding insects inducing defences mainly through the SA pathway (Fidantsef *et al.*, 1999; Walling, 2000; Moran and Thompson, 2001; Chapter 7). Both differences in wounding and differences in herbivory-derived elicitors are likely to be involved in such a differential induction of JA and SA pathways by different herbivore species.

Clearly, JA and SA can induce many responses, and the outcome of induced JA and SA levels can be dependent on the signal-transduction downstream of JA and SA. Mainly through *Arabidopsis* mutant screening, several factors acting downstream of JA and SA are now known (Glazebrook, 2001). Two of these are JAR1 and NPR1. Studies using *jar1 Arabidopsis* mutants, that are insensitive to JA, demonstrated that the *JAR1* locus is involved in signalling downstream of JA and as such is required for resistance mechanisms against many pathogen species, such as resistance against the fungus *Pythium irregulare* and ISR (Staswick *et al.*, 1998; Pieterse *et al.*, 2001b). Studies using *npr1* (=*nim1*) *Arabidopsis* mutants demonstrated that the *NPR1* gene, encoding a transcription-factor binding protein, is involved in signalling downstream of JA and SA and as such is essential for both JA-related ISR and SA-related SAR (Dong, 2001; Pieterse *et al.*, 2001b).

Several authors have pointed out the benefits of genetic approaches in unravelling plant defence mechanisms against herbivores (Mitchell-Olds *et al.*, 1998; Kessler and Baldwin, 2002, Dicke *et al.*, 2002). As *Arabidopsis* is the most studied plant species with respect to genetic dissection of signal transduction pathways, we introduced the model plant *Arabidopsis thaliana* in studies on indirect defence mechanisms. *Arabidopsis* infested with *Pieris rapae* caterpillars emitted a volatile blend that was distinct from mechanically damaged and undamaged plants. Based on these differences in volatile blends, the parasitoid *Cotesia rubecula* preferred *P. rapae*-infested to mechanically damaged and undamaged plants (Chapter 3). The value of a genetic approach for studying indirect defence mechanisms using *Arabidopsis* has been recently demonstrated. When infested with *P. rapae*, transgenic *NahG* plants that cannot accumulate SA emitted a volatile blend that is different from that of infested wild-type plants, resulting in a reduced attraction of *C. rubecula* by infested *NahG* plants (Chapters 4 and 5). Here, we further exploit the combination of a genetic approach and behavioural observation of parasitoids. Using both regurgitant treatment and *jar1-1* and *npr1-1* mutants we demonstrate that herbivore-derived elicitors are involved in herbivory-induced parasitoid attraction of *Arabidopsis* and that the signal transduction pathways involved are distinct from signalling pathways involved in ISR and SAR.

2. Materials and methods

2.1 Plants

Arabidopsis thaliana ecotype Columbia (Col-0), the mutant *jar1-1* line (*Arabidopsis thaliana* ecotype Col-0 mutant with a decreased sensitivity to methyl jasmonate; Staswick *et al.*, 1992) and the mutant *npr1-1* line (*Arabidopsis thaliana* ecotype Col-0 mutant with no expression of pathogenicity related PR-genes; Cao *et al.*, 1994) were grown from seed in a greenhouse (20-30°C, 50-70% r.h., L8:D16). A few days prior to the experiments, 8 to 10-week-old plants were transferred to a climate room (23±1°C, 50-70% r.h., 10 kLux, L8:D16). All plants used were in the vegetative state.

2.2 Insects

Pieris rapae was reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Icarus) in a climate room (21±1°C, 50-70% r.h., 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% r.h., L16:D8). For bioassays, *C. rubecula* pupae were collected and transferred to a gauze cage in a climate room (23±1°C, 50-70% r.h., 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.

2.3 Plant treatments

Caterpillar-infested plants were obtained by placing five (*jar1-1* and *npr1-1* experiments) or twenty (regurgitant experiment) 1st instar *P. rapae* larvae on each plant. The larvae had fed for 24 h on the plants before these plants were used in experiments.

Artificially damaged plants were obtained by rubbing 10 leaves with carborundum powder no. 180 (Cats Import, Hoogvliet, The Netherlands) on a moist cotton pad, 24 h before an experiment. Regurgitant-treated plants were obtained by pipetting 1 µl caterpillar regurgitant on each artificially damaged leaf from an artificially damaged plant immediately after damaging the leaf with carborundum powder. This resulted in 10 µl regurgitant per plant, and as eight plants were used per odour source in the bioassay, 80 µl regurgitant per odour source, which is comparable with the amount of regurgitant used by Mattiacci *et al*. (1994). Regurgitant was collected as described by Mattiacci *et al.* (1994) from 5th instar *Pieris brassicae* larvae.

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.

2.4 Bioassay

Parasitoid two-choice flight experiments were conducted in a wind tunnel set-up (25±5°C, 50-70% r.h., 0.7 kLux) described by Geervliet *et al.* (1994), that was modified according to Chapter 3.

For the flight experiments, two odour sources were placed at the upwind end of the wind tunnel. Each odour source consisted of 8 *Arabidopsis* plants, all having received the same treatment. One day before a bioassay, 4 to 7-days-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.

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 '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 6-10 wasps per pairwise comparison per day.

Choices between two odour sources in the bioassays were statistically analysed using the binomial test. Differences among the percentages of wasps making a choice (responsiveness) were tested using a contingency table test on the absolute numbers.

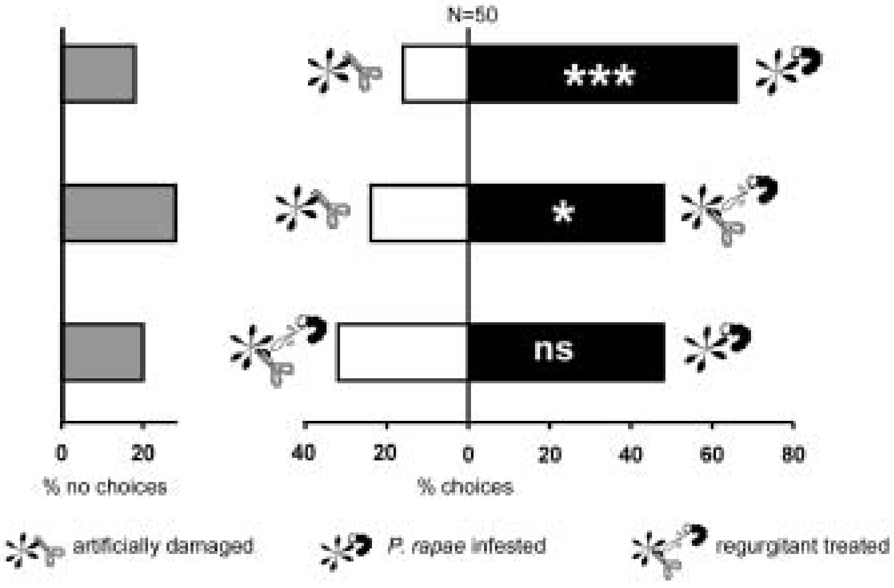


Figure 1. Fggf dupgsf hvshjubouusf bun f oupg *Arabidopsis* po ui f sf t ppot f pg *C. rubecula* jo b ux p di pjdf t f uvp. Nbjwf *C. rubecula* gf n bmf t x f sf hjwf o b di pjdf cf ux f f o *P. rapae*. jogf t uf e boe bsujgjdjbmmz ebn bhf e pmbout - sf hvshjubou usf buf e boe bsujgjdjbmmz ebn bhf e pmbout - boe *P.* sbpbf .jogf t uf e boe sf hvshjubou usf buf e pmbout . Ti f mfgu pbsu pg ui f gjhvsf t i px t ui f pf sdf oubhf pg x bt pt ui bu e je opu mboe po f jui f s pg ui f ux p pepvs t pvsdf t . At uf sjt l t joe jdbuf b t jhojgjdbou e jggf sf odf x jui jo b di pjdf uf t u, *** P<1.112- * P<1.16)cjopn jbmf t u); "b" joe jdbuf t op t jhojgjdbou e jggf sf odf jo ui f ovn cf s pg x bt pt ui bu e je opu mboe po f jui f s pg ui f ux p pepvs t pvsdf t)dpoujohf ody ubcmf uf t u).

3. Results

3.1 Regurgitant treatment induces *C. rubecula* attraction

To test whether herbivore-derived elicitors are involved in herbivory-induced attraction of *C. rubecula* by *Arabidopsis*, we compared mechanically damaged, regurgitant-treated and *P. rapae*-infested plants in a two-choice windtunnel test. *P. rapae*-infested wild-type *Arabidopsis* attracted more *C. rubecula* parasitoid wasps than artificially damaged plants (binomial test, $P<0.001$; Fig. 1). Artificially damaged plants treated with regurgitant also attracted more wasps than plants that were only artificially damaged (binomial test, $P=0.033$; Fig. 1). When *P. rapae*-infested plants were compared with regurgitant treated plants, more wasps landed on *P. rapae*-infested plants, but this was not significant (binomial test, $P=0.134$; Fig. 1). The number of wasps not making a choice was not different between the three comparisons (contingency table test, $P=0.44$).

3.2 *P. rapae*-infested *jar1-1* mutants are equally attractive as *P. rapae*-infested wild-type plants

To test whether signal-transduction in herbivory-induced attraction of *C. rubecula* requires an intact *JAR1* locus, we compared *P. rapae*-infested *jar1-1* mutant plants with *P. rapae*-infested wild-type plants. Both *P. rapae*-infested wild-

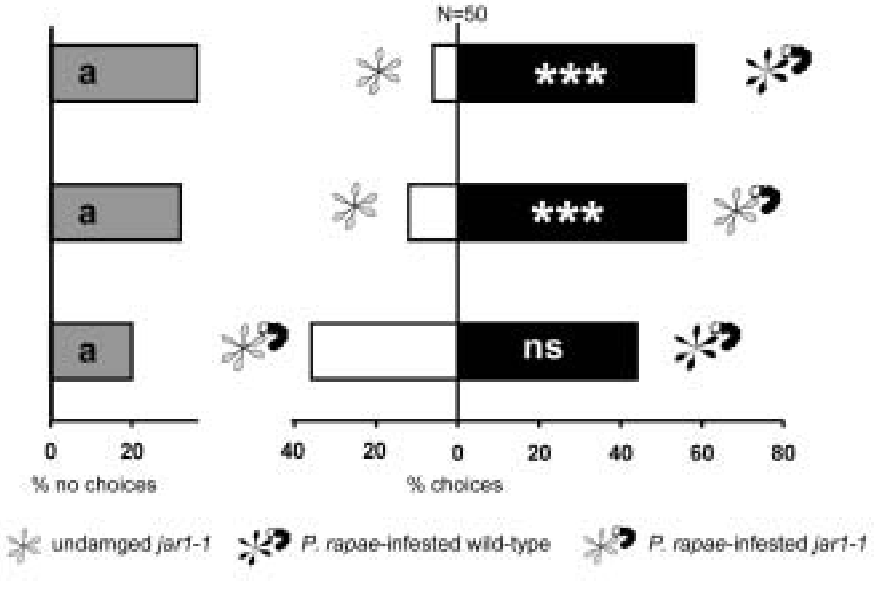


Figure 2. Sftppotf pg *C. rubecula* up *P. rapae*.jogftufe xjme.uypf boe *jar1-1* pmbout. Nbjwf *C. rubecula* gfnbmft xfsf hjwfo b dipjdf cfuxffo *P. rapae*.jogftufe xjme.uypf boe voebnbhfe *jar1-1* pmbout- *P. rapae*.jogftufe *jar1-1* boe voebnbhfe *jar1-1* pmbout- boe *P. rapae*.jogftufe xjme.uypf boe *P. rapae*.jogftufe *jar1-1* pmbout. Tif mfgu pbsu pg uif gjhvsf tipxt uif pfsdfoubhf pg xbtpt uibu eje opu mboe po fjuifs pg uif uxp pepvs tpvsdft. Atufsjtlt joejdbuf b tjhojgjdbou ejggfsfodf xjuijo b dipjdf uftu; *** P<1.112)cjopnjbm uftut); “b” joejdbuft op tjhojgjdbou ejggfsfodf jo uif ovncfs pg xbtpt uibu eje opu mboe po fjuifs pg uif uxp pepvs tpvsdft)dpoujohfodz ubcmf uftut).

type and *iar1-1* plants are more attractive compared to undamaged *iar1-1* plants (binomial test, P<0.001 for both comparisons; Fig. 2). This demonstrates that *P. rapae*-damage can still induce parasitoid attraction in *iar1-1* mutants. Moreover, *C. rubecula* females did not prefer *P. rapae*-infested wild-type over *P. rapae*-infested *iar1-1* plants (binomial test, P=0.32; Fig. 2). The number of wasps not making a choice was not different between the three comparisons (contingency table test, P=0.19).

3.3 P. rapae-infested npr1-1 mutants are equally attractive as P. rapae-infested wild-type plants

To test whether signal-transduction in herbivory-induced attraction of *C. rubecula* requires an intact NPR1 protein, we compared *P. rapae*-infested *npr1-1* mutants with *P. rapae*-infested wild-type plants. Both *P. rapae*-infested wild-type and *npr1-1* plants are more attractive than undamaged *npr1-1* plants (binomial test, P=0.012 and P<0.001 respectively; Fig. 2). This demonstrates that *P. rapae* damage can still induce parasitoid attraction in *npr1-1* mutants. Moreover, *C. rubecula* females did not prefer *P. rapae*-infested wild-type over *P. rapae*-infested *npr1-1* plants (binomial test, P=0.50; Fig. 2). The number of wasps not making a choice was not different between the three comparisons (contingency table, P=0.30).

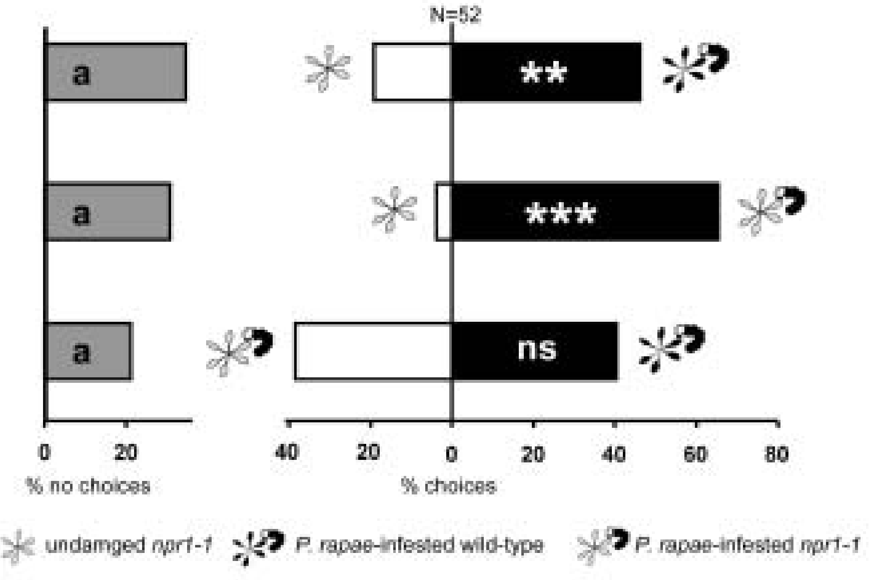


Figure 3. Retppote pf *C. rubecula* tp *P. rapae*-iofettee wile-type boe *npr1-1* plbott. Nbive *C. rubecula* fenblet wese hiveo b dipide betweeo *P. rapae*-iofettee wile-type boe uoebnbhee *npr1-1* plbott- *P. rapae*-iofettee *npr1-1* boe uoebnbhee *npr1-1* plbott- boe *P. rapae*-iofettee wile-type boe *P. rapae*-iofettee *npr1-1* plbott. Tie left pbst pf tie fihuse tipwt tie pesdeotbhe pf wbtpt tibt eie opt lboe po eities pf tie twp pepus tpusdet. Attesitlt ioeidbte b tihoifidbot eiffeseode witiio b dipide tett: *** P<0.001- * P<0.05 (biopn ibl tett); "b" ioeidbtet op tihoifidbot eiffeseode io tie oun bes pf wbtpt tibt eie opt lboe po eities pf tie twp pepus tpusdet (dpotiobeody tbble tett).

4. Discussion

Signal transduction pathways involved in herbivory-induced *indirect* defence overlap with signal transduction pathways involved in other induced defences, such as herbivory-induced direct defence and wound-induced responses and pathogen-induced defence. Wounding induces many responses, including increased production of proteinase inhibitors and toxins involved in defence against herbivores and pathogens (Karban and Baldwin, 1997; Chapter 2). In the current consensus, the most important signalling pathway in wound-induced expression of defence genes is the octadecanoid pathway, with plant hormones such as JA and OPDA as signalling compounds (De Bruxelles and Roberts, 2001; Léon *et al.*, 2001; Stinzi *et al.*, 2001). Similar to wounding, herbivory by chewing insects induces JA levels and subsequent expression of defence genes (Karban and Baldwin, 1997). Moreover, plants that are disabled in the octadecanoid pathway are more susceptible to herbivory (Howe *et al.*, 1996; McConn *et al.*, 1997). However, not all responses to wounding and herbivory are similar. This is likely due to the presence of herbivore-derived elicitors. Indeed, several of these elicitors have been found in the regurgitant of caterpillars (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Halitschke *et al.*, 2001).

In a previous paper, we have demonstrated that herbivory by *P. rapae* and mechanical damage (wounding) induce the emission of different blends of volatiles by *Arabidopsis*. Females of the parasitoid species *C. rubecula* preferred *P. rapae*-infested over mechanically damaged plants (Chapter 3). Although this indicates that the response of *Arabidopsis* with respect to volatile emissions differs between herbivory and wounding, no decisive conclusion could be drawn, as it is very difficult to mimic the mechanical part of herbivore damage (Baldwin, 1990). Here we demonstrate that herbivore-derived elicitors are involved in this induced attraction (Fig. 1). These results show that in *Arabidopsis* there are differences between signalling in wound responses and responses to herbivory. As similar differences have been found in other plant species (Turlings *et al.*, 1990; Steinberg *et al.*, 1993; Mattiacci *et al.*, 1994; Takabayashi *et al.*, 1995), this implies that *Arabidopsis* can function as a suitable model in studies on signal-transduction in indirect defence (see also Chapter 7).

With respect to this signal-transduction, we know that the induction of volatile emission in *Arabidopsis* by herbivory involves both the octadecanoid and salicylic acid pathway. Treatment of plants with JA induces both volatile emissions and parasitoid attraction and plants that do not accumulate SA produce emitted a different volatile blend upon herbivory and are less attractive to parasitoids compared to wild-type plants (Chapters 4 and 5). The *jar1-1* (=*jin4*) mutant is less sensitive to JA with respect to JA-induced inhibition of root growth (Staswick *et al.*, 1992). This JA signalling mutant showed reduced induction of *VSP* genes that are wound-, herbivory-, and JA-inducible (Berger *et al.*, 1996; Berger *et al.*, 2002), was more susceptible to the fungus *Pythium irregulare* compared to wild-type plants (Staswick *et al.*, 1998), and does not show ISR (Pieterse *et al.*, 1998). However, *P. rapae*-infested *jar1-1* mutants showed normal attraction of *C. rubecula* (Fig. 2). This indicates that either the *jar1-1* mutant is leaky or that JA-dependent but *JAR1*-independent pathways are involved. That VSP gene expression is still induced in *jar1-1*, and that *jar1-1* in contrast to other JA-signalling or JA-producing mutants, such as *coi1* and a *fad*-triple mutant, is not male-sterile support these possibilities (McConn and Browse, 1996; Creelman, 1998; Staswick *et al.*, 1998).

Another mutant, *npr1,* is involved in both JA-dependent and SA-dependent signalling. *Npr1* mutants do not show ISR or SAR and SAR-related genes are not induced by biotrophic pathogens (Cao *et al.*, 1994; Pieterse *et al.*, 1998). However, *npr1-1* mutants also showed normal attraction of *C. rubecula* (Fig. 3). Apparently, the JA and SA pathways involved in indirect defence of *Arabidopsis* against *P. rapae* are NPR1-independent. Such an NPR1-independent defence mechanism is not an exception. For example, expression of the defence genes *PDF1.2, PR-3* and *PR-4* and resistance against the fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* is not affected in the *npr1-1* mutant or in transgenic *nahG* plants but is strongly reduced in the JA signalling mutant *coi1-1* (Thomma *et al.*, 1998). This demonstrates the existence of JA-dependent signalling pathways that are NPR1-independent. The *ssi1* mutant constitutively expressed the defence genes *PDF1.2*, *PR-1, PR-2* and *PR5* and showed enhanced resistance against *Pseudomonas syringae* pv tomato. These responses were NPR1-independent as the double mutant *ssi1-npr1-5* showed the same responses as the *ssi1* single mutant, but were SA-dependent as they were suppressed in *ssi1-nahG* plants. The latter suppression was relieved by treatment with the SA-analogue BTH (Shah *et al.*, 1999). This demonstrates the existence of SA-dependent NPR1-independent signalling pathways.

Clearly the signal transduction required for indirect defence against herbivores differs from the signal transduction required for direct defence response against pathogens such as ISR and SAR (Fig. 4). This may not seem surprising, as defences against pathogens and herbivores can be very different. For example, a hypersensitivity response that results in cell death around the place of infection is effective in preventing the spread of pathogens but not against mobile herbivores such as caterpillars (Kessler and Baldwin, 2002). Defence responses induced by infection with the pathogen tobacco mosaic virus (TMV) reduced resistance against the insect herbivore *M. sexta* in tobacco (Preston *et al.*, 1999). This indicates that defence against insects and pathogens can be negatively correlated. However, JA and SA play key roles in the defences against both insects and pathogens and therefore the signalling pathways in these induced defences overlap. Indeed, defences in tomato induced by *P. syringae* pv tomato are also effective against *Helicoverpa zea* caterpillars and vice versa (Stout *et al.*, 1999). Clearly cross-talk and overlap between defence responses against both insects and herbivores can have synergistic and antagonistic effects, depending on plant, insect and pathogen species (Felton and Korth, 2000; Pieterse *et al.*, 2001a). This indicates that signalling mutants downstream of JA and SA potentially affect both defences against insects and pathogens. Indeed, it was recently demonstrated that *npr1-1*

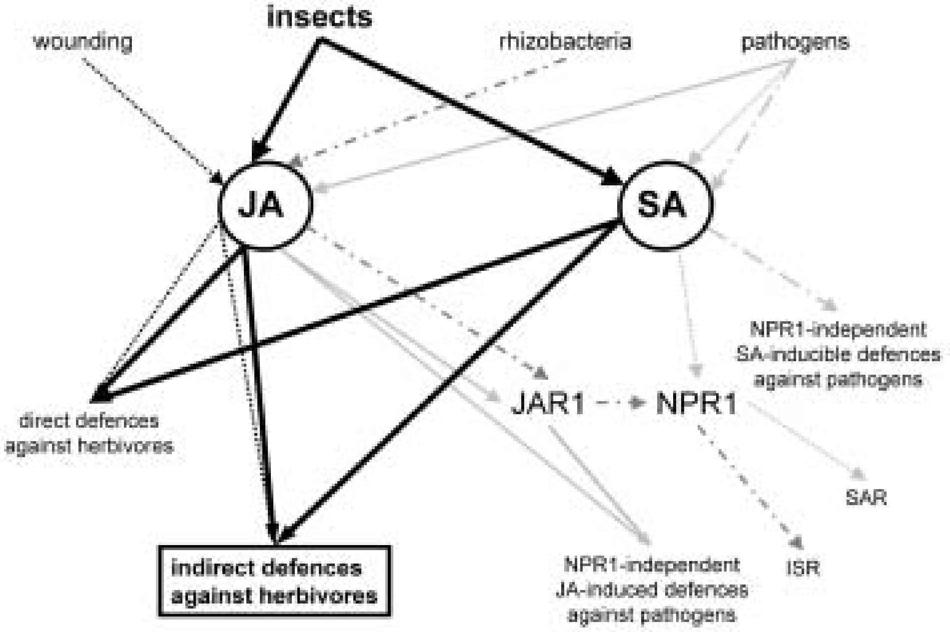


Figure 4. *Proposed scheme for JA- and SA-dependent signal transduction in plant defence. Dotted black lines indicate pathways involved in wound-induced responses, solid black lines pathways of herbivory-induced responses, dark grey lines the pathway of rhizobacteria-induced responses and light grey lines the pathways of pathogen-induced responses.*

mutants showed enhanced resistance against the generalist caterpillar *Spodoptera littoralis* (Stotz *et al.*, 2002). Whether this means that in *Arabidopsis* direct and indirect defence mechanisms against caterpillars are induced through separate signal-transduction pathways, or that generalist caterpillars such as *S. littoralis* and specialists such as *P. rapae* induce different signal-transduction pathways, remains to be investigated. Additionally, it will be interesting to see how other signal-transduction mutants, such as the *coi1* mutants that are more susceptible to pathogen and insect species (Thomma *et al.*, 1998; Xie *et al.*, 1998), are affected in the attraction of parasitoids.

Other plant hormones are also likely to be involved in indirect defence mechanisms. For example, although resistance against *Bradysia impatiens* larvae is reduced in the JA-signalling mutant *coi1*, the production of JA is not necessary for defence against this herbivore. Likely, the JA precursor OPDA plays a key role in this defence (Stintzi *et al.*, 2001). In tobacco plants, feeding by *M. sexta* induces ethylene production that suppresses the accumulation of the defensive compound nicotine, but has no effect on the emission of volatile terpenoids (Kahl *et al.*, 2000). Through which pathways JA and SA influence herbivory-induced volatile production, how these pathways are related to other plant defences such as those against pathogens and which other plant hormones are involved remain intriguing questions. Here we demonstrated that signal-transduction mutants of *Arabidopsis* could provide helpful tools to find the answers.

Acknowledgments

We thank Casper Pillen and Aart van Ommeren for rearing of Arabidopsis plants, and Leo Koopman, Frans van Aggelen and Andre Gidding for the insect rearing. M.D. was partly funded by the Uyttenboogaart-Eliasen Foundation, Amsterdam.

References

Alborn, T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H., Tumlinson, J.H., 1997. An elicitor of plant volatiles from beet armyworm oral secretion. *Science*, 276: 945-949.

Baldwin, I.T., 1990. Herbivory Simulations in Ecological Research. *Trends in Ecology & Evolution*, 5: 91-93.

Berger, S., 2002. Jasmonate-related mutants of *Arabidopsis* as tools for studying stress signaling. *Planta*, 214:497-504.

Berger, S., Bell, E., Mullet, J.E., 1996. Two methyl jasmonate-insensitive mutants show altered expression of AtVsp in response to methyl jasmonate and wounding. *Plant Physiology*, 111: 525-531.

Berger, S., Mitchell-Olds, T., Stotz, H.U., 2002. Local and differential control of vegetative storage protein expression in response to herbivore damage in *Arabidopsis thaliana*. *Physiologia plantarum* 114:85-91.

Cao, H., Bowling, S.A., Gordon, A.S., Dong, X.N., 1994. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell*, 6: 1583-1592.

Creelman, R.A., 1998. Jasmonate perception: characterization of COI1 mutants provides the first clues. *Trends in Plant Science*, 3: 367-368.

De Bruxelles, G.L., Roberts, M.R., 2001. Signals regulating multiple responses to wounding and herbivores. *Critical Reviews in Plant Sciences*, 20: 621-621.

Dicke, M., 1999. Evolution of induced indirect defense of plants. In: R. Tollrian and C.J. Harevell (eds), *The Ecology And Evolution Of Inducible Defenses*. Princeton University Press, Princeton, New Jersey, pp.62-88.

Dicke, M., Van Poecke, R.M.P. and de Boer, J.G. (2002) Inducible indirect defence of plants: from subcellular mechanisms to ecological functions. *Basic and Applied Ecology*, in press.

Dong, X.N., 2001. Genetic dissection of systemic acquired resistance. *Current Opinion in Plant Biology*, 4: 309-314.

Felton, G.W., Korth, K.L., 2000. Trade-offs between pathogen and herbivore resistance. *Current Opinion in Plant Biology*, 3: 309-314.

Felton, G.W., Korth, K.L., Bi, J.L., Wesley, S.V., Huhman, D.V., Mathews, M.C., Murphy, J.B., Lamb, C., Dixon, R.A., 1999. Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. *Current Biology*, 9, 317-320.

Fidantsef, A.L., Stout, M.J., Thaler, J.S., Duffey, S.S., Bostock, R.M., 1999. Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology*, 54: 97-114.

Geervliet, J.B.F., Vet, L.E.M., Dicke, M., 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomologia Experimentalis et Applicata*, 73: 289-297.

Glazebrook, J., 2001. Genes controlling expression of defense responses in *Arabidopsis* - 2001 status. *Current Opinion in Plant Biology*, 4: 301-308.

Halitschke, R., Schittko, U., Pohnert, G., Boland, W., Baldwin, I.T., 2001. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host

Nicotiana attenuata. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiology*, 125: 711-717.

Hartley, S.E., Lawton, J.H., 1991. Biochemical aspects and significance of the rapidly induced accumulation of phenolics in birch foliage. In: D.W. Tallamy and M.J. Raupp (eds), *Phytochemical induction by herbivores*. John Wiley, New York, USA, pp. 105-132.

Howe, G.A., Lightner, J., Browse, J., Ryan, C.A., 1996. An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. *Plant Cell*, 8: 2067-2077.

Kahl, J., Siemens, D.H., Aerts, R.J., Gabler, R., Kuhnemann, F., Preston, C.A., Baldwin, I.T., 2000. Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. *Planta*, 210: 336-342.

Karban, R., Baldwin, I.T., 1997. *Induced Responses to Herbivory*. The University of Chicago Press Ltd, Chicago, USA.

Kessler, A., Baldwin, I.T., 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, 291: 2141-2144.

Korth, K.L., Dixon, R.A., 1997. Evidence for chewing insect-specific molecular events distinct from a general wound response in leaves. *Plant Physiology*, 115: 1299-1305.

Léon, J., Rojo, E., Sanchez-Serrano, J.J. 2001. Wound signalling in plants. *Journal of Experimental Botany*, 52:1-9.

Mattiacci, L., Dicke, M., Posthumus, M.A., 1994. Induction of parasitoid attracting synomone in Brussels sprouts plants by feeding of *Pieris brassicae* larvae: role of mechanical damage and herbivore elicitor. *Journal of Chemical Ecology*, 20: 2229-2247.

Mattiacci, L., Dicke, M., Posthumus, M.A., 1995. beta-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America*, 92: 2036-2040.

McCloud, E.S., Baldwin, I.T., 1997. Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*. Planta, 203: 430-435.

McConn, M., Browse, J., 1996. The critical requirement for linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis* mutant. *Plant Cell*, 8: 403-416.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., Browse, J., 1997. Jasmonate is essential for insect defense Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 94: 5473-5477.

Mitchell-Olds, T., Gershenzon, J., Baldwin, I.T., Boland, W., 1998. Research focus - Chemical ecology in the molecular era. *Trends in Plant Science*, 3: 362-365.

Moran, P.J., Thompson, G.A., 2001. Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. *Plant Physiology*, 125: 1074-1085.

Musser, R.O., Hum-Musser, S.M., Eichenseer, H., Peiffer, M., Ervin, G., Murphy, J.B., Felton, G.W., 2002. Caterpillar saliva beats plant defences: A new weapon emerges in the evolutionary arms race between plants and herbivores. *Nature*, 416: 599-600

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N., Ohashi, Y., 1998. Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant and Cell Physiology*, 39: 500-507.

Ozawa, R., Arimura, G., Takabayashi, J., Shimoda, T., Nishioka, T., 2000. Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. *Plant and Cell Physiology*, 41: 391-398.

Penninckx, I.A.M.A., Thomma, B.P.H.J., Buchala, A., Métraux, J.-P., Broekaert, W.F., 1998. Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell* 10: 2103-2113.

Pieterse, C.M.J., Ton, J., Van Loon, L.C., 2001a. Cross-talk between plant defence siganlling pathways: boost or burden? *AgBiotechNet*, 3: 1-8.

Pieterse, C.M.J., vanWees, S.C.M., vanPelt, J.A., Knoester, M., Laan, R., Gerrits, N., Weisbeek, P.J., vanLoon, L.C., 1998. A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell*, 10: 1571-1580.

Pieterse, C.M.J., Van Pelt, J.A., Van Wees, S.C.M., Ton, J., Leon Kloosterziel, K.M., Keurentjes, J.J.B., Verhagen, B.W.M., Knoester, M., Van der Sluis, I., Bakker, P., Van Loon, L.C., 2001b.

Rhizobacteria-mediated induced systemic resistance: Triggering, signalling and expression. *European Journal of Plant Pathology*, 107: 51-61.

Preston, C.A., Lewandowski, C., Enyedi, A.J., Baldwin, I.T., 1999. Tobacco mosaic virus inoculation inhibits wound-induced jasmonic acid-mediated responses within but not between plants. *Planta*, 209: 87-95.

Reymond, P., Weber, H., Damond, M., Farmer, E.E., 2000. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell*, 12: 707-719.

Shah, J., Kachroo, P., Klessig, D.F., 1999. The *Arabidopsis* ssi1 mutation restores pathogenesis-related gene expression in npr1 plants and renders defensin gene expression salicylic acid dependent. *Plant Cell*, 11: 191-206.

Staswick, P.E., Su, W.P., Howell, S.H., 1992. Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proceedings of the National Academy of Sciences of the United States of America*, 89: 6837-6840.

Staswick, P.E., Yuen, G.Y., Lehman, C.C., 1998. Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus *Pythium irregulare*. *Plant Journal*, 15: 747-754.

Steinberg, S., Dicke, M., Vet, L.E.M., 1993. Relative importance of infochemicals from first and second trophic level in long-range host location by the larval parasitoid *Cotesia glomerata*. *Journal of Chemical Ecology*, 19: 47-59.

Stintzi, A., Weber, H., Reymond, P., Browse, J., Farmer, E.E., 2001. Plant defense in the absence of jasmonic acid: The role of cyclopentenones. *Proceedings of the National Academy of Sciences of the United States of America*, 98: 12837-12842.

Stotz, H.U., Koch, T., Biedermann, A., Weniger, K., Boland, W., Mitchell-Olds, T., 2002. Evidence for regulation of resistance in *Arabidopsis* to Egyptian cotton worm by salicylic and jasmonic acid signaling pathways. *Planta*, 214: 648-652.

Stout, M.J., Fidantsef, A.L., Duffey, S.S., Bostock, R.M., 1999. Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology*, 54: 115-130.

Takabayashi, J., Takahashi, S., Dicke, M., Posthumus, M.A., 1995. Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. *Journal of Chemical Ecology*, 21: 273-287.

Thomma, B., Eggermont, K., Penninckx, I., Mauch Mani, B., Vogelsang, R., Cammue, B.P.A., Broekaert, W.F., 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, 95: 15107-15111.

Turlings, T.C.J., Tumlinson, J.H., Lewis, W.J., 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science*, 30: 1251-1253.

Vijayan, P., Shockey, J., Lévesque, C.A., Cook, R.J., Browse, J. 1998. A role for jasmonate in pathogen defense of *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*. 95: 7209-7214.

Walling, L.L., 2000. The myriad plant responses to herbivores. *Journal of Plant Growth Regulation*, 19: 195-216.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., Turner, J.G., 1998. COI1: An Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science*, 280: 1091-1094.

chapter

6

Attraction of the specialist parasitoid *Cotesia rubecula* to *Arabidopsis thaliana* infested by host or non-host herbivore species

Remco M.P. van Poecke, Mara Roosjen, Lorena Pumarino, and Marcel Dicke

"'Op de Champs Elysées,' vervolgde hij, 'wat toch een behoorlijk brede verkeersader is, kan mijn neus met grote nauwkeurigheid vaststellen welk parfum een vrouw gebruikt, die aan de overkant van de straat loopt.'
'Met verkeer tussen u in?'
'Met drúk verkeer tussen ons in,' zei hij."

Roald Dahl - Teef

Chapter 7

ATTRACTION OF THE SPECIALIST PARASITOID *Cotesia rubecula* TO *Arabidopsis thaliana* INFESTED BY HOST OR NON-HOST HERBIVORE SPECIES

Abstract

In this study we investigated whether in a two-choice set-up the parasitoid *Cotesia rubecula* (Marshall) can distinguish between *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) infested with its host, *Pieris rapae* (L.) (Lepidoptera: Pieridae) and *Arabidopsis* infested with non-host herbivores. Four non-host herbivore species were tested: the caterpillars *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), both chewing insects; the spider mite *Tetranychus urticae* (Koch) (Acari: Tetranychidae) that punctures parenchymal cells; and the aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphidoidea), that is a phloem-feeder. Compared with undamaged plants, *C. rubecula* females were more attracted to *Arabidopsis* plants infested by *P. rapae*, *P. xylostella*, *S. exigua* and *T. urticae*, but not to plants infested by *M. persicae*. The parasitoids did prefer host-infested to spider mite- or aphid-infested plants, but not to plants infested with non-host caterpillars (*P. xylostella* or *S. exigua*). Apparently, when *Arabidopsis* plants are infested with a leaf tissue-damaging herbivore they emit a volatile blend that is attractive to *C. rubecula* females and the wasps only discriminate between a host and non-host herbivore when the type of damage is different (chewing versus piercing). When *Arabidopsis* is infested with a herbivore that hardly damages leaf tissue, *C. rubecula* females are not attracted. How this relates to plant hormones involved in the induction of plant defence and to results obtained with other plant species is discussed.

1. Introduction

In a natural environment, plants are exposed to many attacks, including those by herbivorous arthropods. To defend themselves against arthropods, plants have developed both constitutive and inducible defence mechanisms. Such inducibility requires information processing in the plant. Several compounds act as signalling compounds in inducible defences, with important roles for salicylic acid (SA) and compounds from the octadecanoid pathway such as jasmonic acid (JA) (see Karban and Baldwin, 1997; Walling, 2000; and Chapter 2 for reviews). Jasmonic acid is involved in wound-induced responses whereas SA is mostly known for its role in induced defences against many pathogens. Damage by chewing insects, that cause substantial wounding, generally induces defences through the octadecanoid pathway, whereas phloem-feeding insects such as aphids induce mainly SA-related defence mechanisms (Karban and Baldwin, 1997; Walling, 2000; Moran and Thompson, 2001).

Besides direct defence mechanisms, plants can also use indirect defence mechanisms (reviewed by Dicke, 1999). Indirect defence mechanisms promote the effectiveness of natural enemies of herbivorous arthropods, i.e. predators or parasitoids. For example, plants produce volatiles upon herbivory that can be used by predators or parasitoids to locate their prey or hosts (Dicke, 1999). Similarly to direct defence mechanisms, both the octadecanoid pathway and the salicylic acid pathway can be involved in herbivory-induced volatile production. Damage inflicted by chewing insects appears to induce volatile emission mainly through the octadecanoid pathway, whereas volatile emission induced by cell-sucking insects such as spider mites appears to involve both the octadecanoid and SA pathway (Dicke dsak-, 1999; Ozawa dsak-, 2000; Kessler and Baldwin, 2001).

As different herbivore species can induce different signal transduction pathways, they can also induce different blends of volatiles emitted by the plant. Some carnivore species can use this information to distinguish between prey-infested and non-prey-infested plants. For instance, starved predatory mites (Ogxsnrdhtkt r odrrhl hkhr) prefer volatiles from Lima bean plants infested by their host, S- t rshbad spider mites, over those infested by Roncnosdra dwhf t a caterpillars (Shimoda and Dicke, 2000). Similarly, the parasitoid wasp Barchmbghkdr mhf rhbdbr prefers tobacco or cotton plants infested by its host, Hdkmsghr uhrdrbdmr caterpillars, to conspecific plants infested by the non-host caterpillar Hdkhbnudroa yda (De Moraes dsak- 1998), and the aphid parasitoid Aoghcht r druh prefers bean plants infested by its host Abxrsgnrhognmohrt l to bean plants infested by the non-host aphid Aoghr eaaad (Du dsak-, 1996).

To get a better understanding of the involvement of different signal-transduction pathways in herbivory-induced volatile production by plants, we recently introduced Araahcnorhr sgakhama in studies on indirect defence (Chapters 3 and 4). Many mutant and transgenic Araahcnorhr plants are available that are altered in the octadecanoid or salicylic-acid signalling pathways. We have demonstrated that B- rt adbt ka is attracted to O- raoad-infested Araahcnorhr and that it can distinguish between O- raoad-infested and mechanically damaged Araahcnorhr (Chapter 3). In this paper we address the response of B- rt adbt ka to Araahcnorhr infested with non-host herbivores. Two of the non-hosts, i.e. Okt sdkka wxknrsdkka and Roncnosdra dwhf t a, have a feeding style that is similar to O- raoad: they both are lepidopteran species that remove large areas of leaf tissue. Okt sdkka wxknrsdkka is a non-host caterpillar with a -plant range similar to that of O- raoad- Both O- wxknrsdkka and O- raoad only feed on plants that contain glucosinolates and are therefore likely adapted to the defences of their host plants (Renwick and Lopez, 1999; Talekar and Shelton, 1993). In contrast, R- dwhf t a is extremely polyphagous (Berdegue ds ak-, 1998). The third non-host species, the spider mite Sdsramxbgt r t rshbad, is a polyphagous herbivorous mite. It feeds by puncturing individual parenchymal cells and sucking the cell contents (Tomczyk and Kropczyńska, 1985). The fourth non-host species, the aphid L xyt r odrrhbad is also polyphagous (Van Emden ds ak- 1969). This species feeds on phloem sap and hardly damages parenchymal cells (Tjallingii and Hogen-Esch, 1993). We hypothesised that Araahcnorhr plants infested with different herbivores induce different signal-transduction pathways and as a consequence emit different volatile blends. To test this we investigated whether the parasitoid B- rt adbt ka can discriminate between volatiles emitted by different herbivore species.

2. Materials and methods

2.1 Plants

Araahcnorhr sgakhama ecotype Columbia (Col-0), the mutant iar0,0 line Araahcnorhr sgakhama ecotype Columbia (Col-0) was grown from seed in a greenhouse (20-30 °C, 50-70% r.h., L8:D16). A few days prior to the experiments, 8 to 10-week-old plants were transferred to a climate room (23±1°C, 50-70% r.h., 10 kLux, L8:D16) where they remained until they were used in the bioassay. All plants used were in the vegetative state.

2.2 Insects

Chdrhr raoad was reared on Brussels sprouts plants (Ararrhba nkdrabda f d l l hedra cv. Icarus) in a climate room (21±1°C, 50-70% r.h., L16:D8).

Okt sdkka wxknrsdkka was also reared on Brussels sprouts in a climate room (25±1°C, 40-50% r.h., L16:D8).

Roncnosdra dwhf t a was reared on an artificial diet in a climate room [27±2°C, 70-80% r.h., L16:D8, see also (Smits ds ak-, 1986)]. For experiments, eggs were transferred to Brussels sprouts leaves in a plastic box and placed in a climate room (23±1°C, 50-70% r.h., L8:D16). After hatching, the first instar caterpillars were used.

Sdsramxbgt r t rshbad was reared on Lima bean plants (Ogarchkt r kt mast r L- cv. Sieva) in a greenhouse (25±5°C, 50-70% r.h., L16:D8).

L xyt r odrrhbad was reared on Chinese cabbage plants (Ararrhba bghmdmrhr L. cv. Granaat) in a greenhouse (25±5°C, 50-70% r.h., L16:D8).

The parasitoid Bnsdrha rt adct ka was reared on O- raoad larvae on Brussels sprouts plants in a greenhouse (25±5°C, 50-70% r.h., L16:D8). For bioassays, B- rt adct ka pupae were collected and transferred to a gauze cage in a climate room (23±1°C, 50-70% r.h., L16:D8). The emerging wasps were provided with water and honey. These adult wasps did not have contact with plant material or caterpillars (no oviposition experience), and are therefore referred to as 'naive' wasps.

2.3 Plant treatments

Chdrhr raoad versus Ckt sdkka wxknrsdkka: Chdrhr raoad,infested *Ara*ahcnorhr plants were obtained by placing ten first or second instar larvae on each plant, O- wxknrsdkka, infested plants were obtained by placing ten second or third instar larvae on each plant. The larvae had fed for 24 h on the plants before these plants were used in experiments. Eight plants per treatment were used.

Chdrhr raoad versus Roncnosdra dwhf t a: Chdrhr raoad-infested *Ara*ahcnorhr plants were obtained by placing ten first instar larvae on each plant, R- dwhf t a,infested plants were obtained by placing 13 first instar larvae on each plant. The larvae had fed for 24 h on the plants before these plants were used in the experiments. Four plants per treatment were used.

Chdrhr raoad versus Sdsramxbgt r t rshbad: Chdrhr raoad,infested *Ara*ahcnorhr plants were obtained by placing two first instar larvae on each plant, S- t rshbad,infested plants were obtained by placing 55 adult females on each plant. The herbivores had fed for seven days on the plants before these plants were used in experiments. Eight plants per treatment were used.

Chdrhr raoad versus L xyt r odrrhbad: Chdrhr raoad,infested *Ara*ahcnorhr plants were obtained by placing two first instar larvae on each plant, L - odrrhbad,infested plants were obtained by placing 15 adult females on each plant. The herbivores had fed for seven days on the plants before these plants were used in experiments. Eight plants per treatment were used.

In all experiments, undamaged (control) plants had not received any treatment but were of the same age and size as the treated plants and had been transferred to the climate room at the same time.

2.4 Bioassay

Parasitoid two-choice flight experiments were conducted in a wind tunnel set-up (25±5°C, 50-70% r.h., 0.7 kLux) described by Geervliet ds ak- (1994), and modified according to Chapter 3. For the flight experiments, two odour sources were placed at the upwind end of the wind tunnel. Each odour source consisted of 4 or 8 *Ara*ahcnorhr plants, all receiving the same treatment. One day before a bioassay, 4-7-days old, naive B- rt adct ka 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 O- raoad-damaged wild-type *Ara*ahcnorhr plant, from which the caterpillars had been removed. The slide, with wasp and leaf, was transported to the centre of the release cylinder in the wind tunnel, which was 60 cm downwind of the odour sources.

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 '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. The experiments were repeated on several days with 4-10 wasps per pairwise comparison per day. Per pairwise comparison, the difference in the total number of wasps landing on or the other odour source was statistically analysed using a binomial test. Using the separate experimental days as replicates, the difference between the numbers of wasps landing on O- *ra*o*a*d-infested or non-host-infested plants was tested using a two-tailed Wilcoxon matched pairs signed ranks test. Differences in the percentage of wasps making a choice (responsiveness) were tested using a contingency table on absolute numbers.

2.5 Damage analysis

To compare the amount of tissue eaten by O- *ra*o*a*d with the amount of tissue eaten by O- wxknrsdkk*a* or R- dWrf t *a*, all leaves of all caterpillar-infested plants used in the bioassays were cut off and taped on a white paper sheet. This sheet was photocopied. Using these photocopies, the area of damage per leaf was traced on a transparent sheet using a coloured permanent marker (see also Chapter 4). 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).

The area of damage (the pooled data of all plants receiving the same treatment per experimental day, i.e. 8 plants per odour source in the O- *ra*o*a*d versus O- wxknrsdkk*a* experiment and 4 plants per odour source in the O- *ra*o*a*d versus R- dWrf t *a* experiment) of two odour sources was compared using a Mann-Whitney U test.

The Spearman rank-order correlation test was performed to test whether per experimental day, differences in attraction of B- *r*t adct k*a* could be related to differences in the amount of damage. The difference in damage between O- *ra*o*a*d infested and non-host (either O- wxknrsdkk*a* or R- dWrf t *a*) infested plants per experimental day was calculated as:

X = DPr - DPn

DPr = the area of damage of O- *ra*o*a*d infested plants

DPn = the area of damage of non-host-infested plants.

The difference in attraction in the direct comparison between O- *ra*o*a*d, infested and non-host-infested *Ara*ahcnorhr (see Ahn*a*rr*a*x) per experimental day was calculated as:

$Y = (Pr - Pn)/N$
Pr = number of wasps choosing O- raoad infested plants
Pn = number of wasps choosing a non-host infested plants
N = total number of wasps tested on that day for that comparison.

3. Results

3.1 *Pieris rapae* versus *Plutella xylostella* (Figure 1A)

Bnsdrha rt adbt ka females clearly preferred plants infested with their host O- raoad over non-infested, undamaged plants (binomial test, $P<0.001$), as was demonstrated before (Chapter 3). Plants infested with non-host caterpillars of the species O- wxknrsdkka were also preferred over undamaged plants (binomial test, $P<0.001$). Moreover, the wasps did not distinguish between host-infested and non-host-infested Araahcnorhr when either the total number of wasps (binomial test, $P=0.14$) or the number of wasps per experimental day (Wilcoxon matched pairs signed rank test, $P=0.35$) was tested. The responsiveness between the three comparisons did not differ (contingency table, $P=0.62$).

After 24 h, the 10 first or second instar O- raoad caterpillars had removed approximately 10% of the leaf tissue. The amount of tissue eaten by 10 second or third instar O- wxknrsdkka caterpillars was 1.5 fold larger (Mann-Whitney-U test, $P=0.049$; Fig 1A, insert). However, when considering the separate experimental days, there was no correlation between differences in amount of feeding and differences in parasitoid attraction (Spearman rank-order correlation, $P>0.1$).

3.2 *Pieris rapae* versus Spodoptera exigua (Figure 1B)

Similar results were obtained when another non-host caterpillar species, R- dwhft a, was used. Host and non-host caterpillar-infested Araahcnorhr were preferred over undamaged plants (binomial test, $P<0.001$ for both comparisons). Again, the wasps did not distinguish between host-infested and non-host-infested Araahcnorhr when either the total number of wasps (binomial test, $P=0.24$) or the number of wasps per experimental day (Wilcoxon matched pairs signed rank test, $P=0.42$) was tested. The responsiveness between the three comparisons did not differ (contingency table test, $P=0.42$).

The amount of tissue eaten by ten first instar O- raoad caterpillars was equal to the amount of tissue eaten by 13 first instar R- dwhft a caterpillars, approximately 5% of the leaf tissue (Mann-Whitney-U test, $P=0.33$; Fig 1B, insert). Moreover, when considering the separate experimental days, there was no correlation between differences in amount of tissue eaten and parasitoid attraction (Spearman rank-order correlation, $P>0.1$).

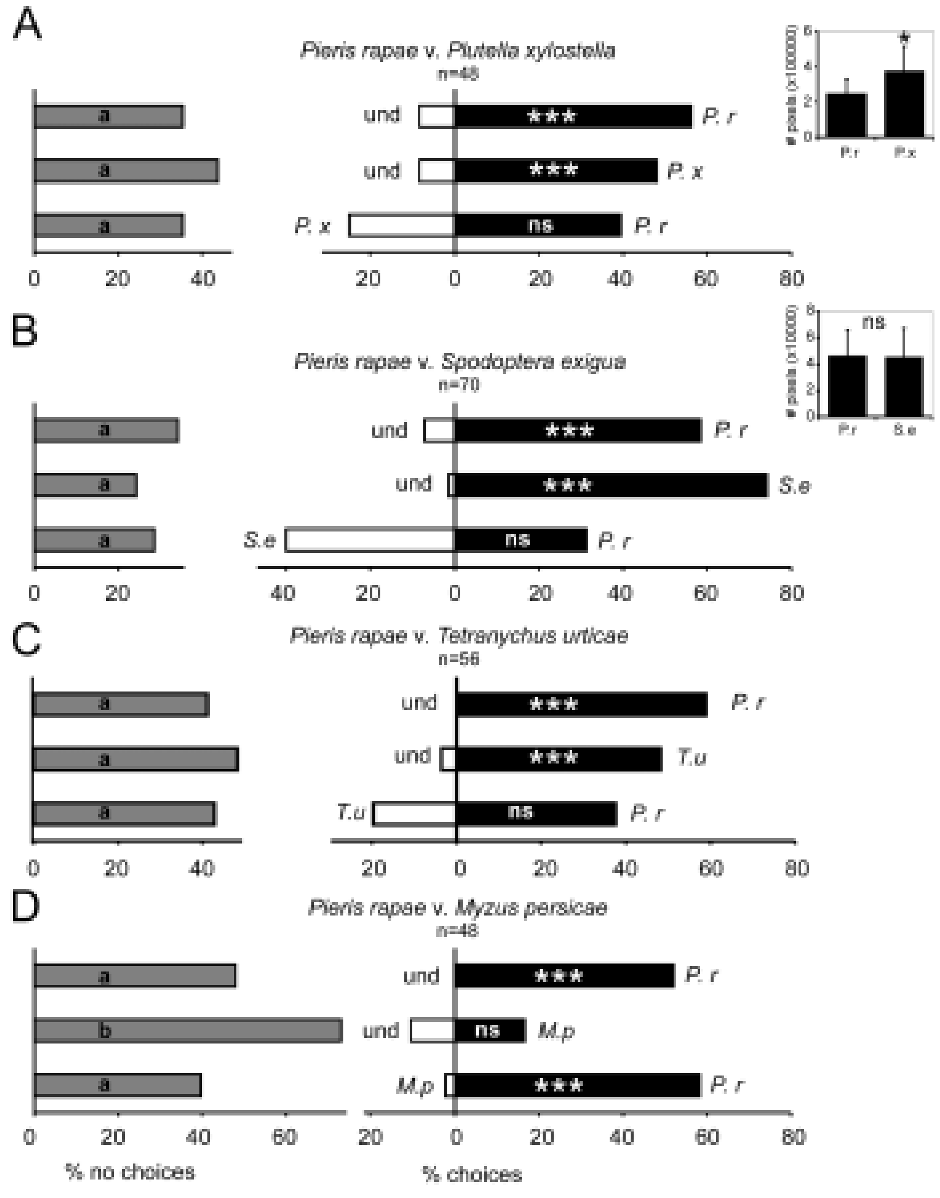


Figure 1. *Sftqpotf pg* *C. rubecula* *up iptujogftufe boe opo.iptujogftufe* *Arabidopsis* *jo b uxp dipjdf tfuvq.* N*bjvf* *C. rubecula* *gfnbmft xfsf hjvfo b dipjdf cfuxffo* *P. rapae.* *jogftufe* (*iptu* *P.r*) *boe voebnbhfe* (*voe*) *Arabidopsis*; *opo.iptu boe voebnbhfe* *Arabidopsis*; *boe iptu boe opo.iptujogftufe* *Arabidopsis*. N*po.iptu ifscjvpsft xfsf* (*B*) *P. xylostella* (*P.x*); (*C*) *S. exigua* (*S.e*); (*D*) *T. urticae* (*T.u*); *boe* (D) *M. persicae* (*M.p*). T*if rhguqbsupg uif gjhvsf tipxt uif qfsdfoubhf pg xbtqt uibu ejeje opumboe po pof pg uif uxp pepvs tpvsdft.* T*if jotfsut jo* (*B*) *boe* (C) *tipx uif bnpvou pg ujttvf bsfb sfnpvfe* (*fyqsfttfe bt qjyfmt; gps efubjmt po nfuipe tff ufyu*) *hspvqfe qfs pepvs tpvsdf.* T*if ovncfs pg xbtqt uftufe qfs dpncjobujpo pg pepvs tpvsdft jt joejdbufe xjui o.* D*jggfsfodf dibsbdufst* (*b ps c*) *joejdbuf b tjhojgjdbou ejggfsfodf jo uif ovncfs pg xbtqt uibu ejeje opu mboe po pof pg uif uxp pepvs tpvsdft* (*dpoujohfodz ubcmf*). *Btufsjtlt joejdbuf b tjhojgjdbou ejggfsfodf xjui jo b dipjdf uftu.* *** P<*1.112* (*cjopnjbmuftu*).

3.3 *P*ieris rapae **versus** Tetranychus urticae **(Figure 1C)**

In 7 days two first instar O- *ra*oad larvae had removed approximately 10% of the leaf tissue. Similar to previous experiments, host-infested plants were more attractive than undamaged plants (binomial test, P<0.001). Plants infested with the non-host herbivorous mite S- t *r*shb*a*d were also more attractive than undamaged plants (binomial test, P<0.001). There was a slight preference for volatiles from host-infested over spider-mite-infested *Ara*ahcnorhr, which was only marginally insignificant using the total number of wasps (binomial test, P=0.055) and which was significant using the number of wasps per experimental day (Wilcoxon matched pairs signed rank test, P=0.047). The responsiveness between the three comparisons did not differ (contingency table, P=0.26).

3.4 *P*ieris rapae **versus** *M*yyus persicae **(Figure 1D)**

Volatiles from *Ara*ahcnorhr infested with the non-host phloem-feeding herbivore-species L xyt r odrrhb*a*d, did not attract B- *r*t adbt k*a*. The wasps did not distinguish between undamaged and L - odrrhb*a*d-infested plants (binomial test, P=0.29), while they did distinguish between undamaged and host-damaged plants (binomial test, P<0.001). Moreover, they clearly preferred O- *ra*oad-infested *Ara*ahcnorhr to L - odrrhb*a*d-infested plants when either the total number of wasps (binomial test, P<0.001) or the number of wasps per individual day (Wilcoxon signed rank test, P=0.016) was tested. This time, there was a difference in responsiveness between the three comparisons, with the combination L - odrrhb*a*d-infested plants versus undamaged plants attracting fewest wasps (contingency table test, P=0.003).

4. Discussion

4.1 The role of plant hormones in herbivore-specific emission of volatiles by Arabidopsis

We investigated whether B- *r*t adbt k*a* can distinguish between volatiles from *Ara*ahcnorhr sg*a*kh*a*m*a* infested by its host, O- *ra*oad, and *Ara*ahcnorhr infested by non-host herbivore species. Leaf-tissue damage of *Ara*ahcnorhr is apparently required for an increased attraction compared to undamaged plants: Infestation with a phloem-feeder did not result in a higher attraction compared to undamaged plants (Figure 1D) but infestation with leaf-feeders such as spider mites or non-host caterpillars did (Figure 1A-C). Indeed, mechanically damaged *Ara*ahcnorhr plants are also more attractive than undamaged plants (Chapter 3). As wounding induces the octadecanoid pathway (including elevated JA levels) in *Ara*ahcnorhr (Bell ds *a*k-, 1995; Reymond ds *a*k-, 2000) it appears that this pathway plays a dominant role in herbivory-induced volatile production (Chapter 4). Such an important role for JA in volatile production induced by leaf tissue-damaging herbivores has been demonstrated in other plant species as well (reviewed in Chapter 2). Spraying *Ara*ahcnorhr with JA resulted in an increased attraction of B- *r*t adbt k*a*. Moreover, *LOX*1-cosuppressed plants that do not show increased JA levels upon wounding are less attractive to B- rt addt ka when infested by O- raoad larvae compared to O-raoad-infested wild-type plants (Chapter 4).

We recently demonstrated that besides the octadecanoid pathway, the salicylic-acid pathway is also involved in caterpillar-induced volatile emission by *Ara*ahcnorhr: Transgenic *Na*gF plants that cannot accumulate SA were less attractive to B- rt addt ka when infested by O- raoad larvae compared to infested wild-type plants. However, spraying plants with SA did not result in an increased attraction of B- rt addt ka (Chapter 4)- This also indicates that induction of the octadecanoid pathway (either through wounding or JA-spraying) is required, which would explain why aphid-infestation of *Ara*ahcnorhr did not result in the attraction of B- rt addt ka. Indeed, Moran and Thompson (2001) demonstrated that aphid infestation resulted in a strong induction of SA-inducible defences but had only a weak effect on JA-inducible defences in *Ara*ahcnorhr and similar results have been found in tomato (Fidantsef ds ak-, 1999). This indicates that although both caterpillar infestation and aphid infestation induce the octadecanoid as well as the salicylic acid-pathway in *Ara*ahcnorhr, caterpillar feeding has a stronger effect on the octadecanoid pathway and aphid feeding has a stronger effect on the salicylic acid pathway. The difference in relative induction between these pathways likely results in a difference in the volatiles emitted. In corn, infestation with stemborers or caterpillars resulted in a different induction of volatile emission, but infestation with aphids did not result in detectable emission of volatiles at all (Turlings ds ak-, 1998).

Spider mite feeding resulted in the attraction of B- rt addt ka (Figure 1C). Although the preference for O- raoad,infested plants compared to S- t rshbad-infested plants was marginally insignificant (binomial test, P=0.055) when using the total number of wasps, we noticed that on 5 out of the 7 experimental days, more B- rt addt ka flew to O- raoad-infested plants than to spider-mite infested plants, whereas on only 1 day S- t rshbad-infested plants were preferred, which is significantly different. In the experiments where B- rt addt ka did not distinguish between O-raoad-infested and O- wxknrsdkka or R- dwhf t a-infested *Ara*ahcnorhr we did not see such a difference. This indicates that B- rt addt ka can distinguish between O- raoad and S- t rshbad-infested plants. Spider mite feeding possibly results in a more equal induction of both the octadecanoid and the salicylic acid pathway. In Lima bean it was found that successive treatment with JA and methyl salicylate (MeSA) resulted in the emission of a volatile blend similar to spider-mite feeding, whereas JA-treatment alone resulted in the emission of a volatile blend similar to caterpillar feeding (Ozawa ds ak-, 2000), suggesting that spider mite feeding results in a stronger activation of the salicylic acid pathway than caterpillar feeding.

4.2 Arabidopsis **as a model plant for indirect defences**

The most important source of variation in odour blends emitted by plants is the plant species (Dicke, 1999). Some plant species, like lima bean and corn, emit volatiles upon herbivory that are hardly or not emitted by undamaged or mechanically damaged plants (Dicke ds ak- 1990; Turlings ds ak- 1990). If infested by different herbivore species they can emit volatile blends that differ quantitatively and/or qualitatively (Turlings ds *a*k- 1998). Other plant species, like Brussels sprouts, emit a volatile blend upon herbivory that is qualitatively similar to the blend from undamaged or mechanically damaged plants, but do so in larger amounts. If these plants are infested with different herbivore species, they can emit volatile blends that are quantitatively different or have minor qualitative differences (Blaakmeer ds *a*k-, 1994; Mattiacci ds *a*k, 1994; Geervliet ds *a*k, 1997). Previously, we demonstrated that, just as in Brussels sprouts, the volatile blend emitted by mechanically damaged *Ara*ahcnorhr shows mainly quantitative differences with the blend emitted by O- *ra*o*a*d-infested *Ara*ahcnorhr (Chapter 3).

Consistent with this, the cruciferous plants studied so far emit volatile blends in response to feeding by different chewing herbivore species that are quite similar. Chemical analyses revealed only minor differences between the volatile blends of O- *ra*o*a*d and O- a*ra*rrhb*a*d-infested Brussels sprouts plants (Blaakmeer ds *a*k-, 1994). Similar results were found for white cabbage, red cabbage and nasturtium with the same two herbivore species (Geervliet ds *a*k-, 1997), for O- *ra*o*a*d and O- wxknrsdkk*a*-infested cabbage (Agelopoulos and Keller, 1994b; Shiojiri ds *a*k- 2001). These results are reflected in the attraction of B- *r*t addt k*a* to these herbivore-damaged plant species. The parasitoid did not distinguish between conspecific plants infested by different lepidopteran species. This was investigated for e.g. Brussels sprouts, red, green or white cabbage, or the non-crucifer nasturtium, infested by different lepidopteran species (Geervliet ds *a*k-, 1996; Agelopoulos and Keller, 1994a). Bnsdrh*a* *r*t addt k*a* did prefer O- *ra*o*a*d-damaged cabbage plants to cabbage damaged by a leaf tissue-damaging non-lepidopteran non-host herbivore (snails) and to mechanically damaged plants (Agelopoulos and Keller, 1994a; Geervliet ds *a*k-, 1996).

All the plant species mentioned above are cultivated plant species, which might have lost their capability to produce herbivore-specific information. However, the response of B- *r*t addt k*a* to *Ara*ahcnorhr plants infested with different herbivore species is similar. The wasps did distinguish between plants infested by their host and plant infested by a non-lepidopteran non-host, but not between plants infested by their host and plants infested with a non-host lepidopteran. Plants infested with O- wxknrsdkk*a* or infested with R- dWhf t *a* were equally attractive compared to O- *ra*o*a*d-infested *Ara*ahcnorhr (Figure 1A,B)- Although the larvae of O- wxknrsdkk*a* had removed more tissue than the larvae of O- *ra*o*a*d (Figure 1A, insert), we found no relation between the differences in damage and the attraction of B- *r*t addt k*a* on separate experimental days. Therefore, it is unlikely that the larger amount of tissue removed by O- wxknrsdkk*a* can explain why B- *r*t addt k*a* did not discriminate between these odour sources. The amount of damage inflicted by the R- dWhf t *a* larvae was the same as the amount of damage by the O- *ra*o*a*d larvae (Figure 1B, insert). Moreover, we found no relation between the differences in damage and the attraction of B- *r*t addt k*a* on separate experimental days. Apparently, B- *r*t addt k*a* does not distinguish between plants infested with its host and plants infested with a herbivore species with a similar way of feeding as its host.

This indicates that cruciferous plants emit a similar volatile blend if infested with herbivore-species with a similar way of feeding and that this is not limited to cultivated plants. Indeed, Benrey ds ak- (1998) demonstrated that cultivated crop plants do not show reduced attraction of the parasitoids B- fknl drasa and Rsdmnbnrrd art bghunra compared to noncultivated relatives, for respectively two crucifer species and three bean species. Together with previous results (Chapter 3), our present data demonstrate that Araahcnorhr is a suitable model-plant for studying indirect defences in crucifers.

4.3 Different parasitoid species and the response to herbivory -induced volatiles

As argued by Dicke and Vet (1999), the different types of plant responses (the emission of quantitative or qualitative different blends) may influence carnivore performance. For example, the parasitoid Ldosnohkhma gdsdrnsnl a could learn to discriminate qualitatively different odour blends but needed additional, unrewarding experiences to learn to discriminate between odour blends with only (small) quantitative differences (Vet ds ak-, 1998). Similarly, naive females of the parasitoid Bnsdrha fknl drasa did not distinguish between Brussels sprouts plants infested by Chdrhr ararrhbad or O- raoad caterpillars, but after multiple oviposition experiences B- fknl drasa preferred plants infested by O- ararrhbad, which is a more suitable host for this parasitoid (Geervliet ds ak-, 1998)- In contrast, B- rt adbt ka females, which are closely related to B- fknl drasa, did not distinguish between O- raoad and O- ararrhbad infested plants, even after multiple oviposition experiences; yet this parasitoid species is a specialist on O- raoad (Geervliet ds ak-, 1998; Harvey ds ak-, 1999). Shiojiri ds ak- (2000) showed that naive B- fknl drasa did not distinguish between O- wxknrsdkka-infested and O- raoad-infested cabbage, but naive B- okt sdkkad did.

This demonstrates that the response of parasitoids towards plant species that emit volatile blends with only minor differences when infested by different caterpillar species depends on the parasitoid species. Apparently, some parasitoid species, such as B- okt sdkkad, use the long-distance herbivore-specific volatiles from plants, others, such as B- fknl drasa, do so only after learning experiences, and some, such as B- rt adbt ka, use this long-distance information as a general cue and can distinguish between host- and non-host infested plants after landing (Geervliet ds ak-, 1998; Vos ds ak-, 1998; Shiojiri ds ak-, 2000). These different strategies do not necessarily originate from different capabilities. A plastic behaviour that can be influenced by learning is more important for generalist parasitoids like B- fknl drasa than for specialist parasitoids like B- rt adbt ka and B- okt sdkkad (Geervliet ds ak-, 1998; Vos ds ak-, 1998). Additionally, Vos ds ak- (2001) argued that persistence of parasitoid species is influenced by the capability to distinguish between volatiles related to hosts and non-hosts and by the diversity of herbivore species present. Depending on the herbivore diversity, parasitoids that waste too much time by being attracted to non-host-infested plants would go extinct, but parasitoids that do not waste any time on non-host-infested plants would go extinct as well. This indicates that the different strategies used by B- rt adbt ka and B- okt sdkkad both have their advantages and disadvantages, which are influenced by the diversity of herbivores present in the field. It would be interesting to see how different parasitoid species respond to volatiles from Araalrcnorhr plants infested with a host or non-host herbivore.

4.4 Conclusions

Our study demonstrates that with respect to the attraction of the parasitoid B-rt addt ka, Araalrcnorhr shows similar responses when infested by different herbivores compared to other crucifers studied so far. This also shows that Araalrcnorhr is an appropriate model to study indirect plant defences. The many mutant and transgenic Araalrcnorhr plants available can help us to get a better understanding of the signal-transduction pathways involved in the responses of Araalrcnorhr induced by different herbivores. We have argued that both the salicylic acid and the octadecanoid pathway are involved in the herbivore-specific responses of Araalrcnorhr. How the balance between these two pathways is regulated and which other signalling factors are involved remains to be investigated.

Acknowledgments

We thank Leo Koopman, Frans van Aggelen and Andre Gidding for the insect rearing, Casper Pillen for rearing of Arabidopsis plants, and Jetske de Boer for comments on this manuscript. M.D. was partly funded by the Uyttenboogaart-Eliasen Foundation, Amsterdam.

References

Agelopoulos, N.G., Keller, M.A., 1994a. Plant-natural enemy association in the tritrophic system Bnsdrha rt addt ka-Chdrhr raoad-Brassicaceae (Cruciferae): II. Preference of B- rt addt ka for landing and searching. Jnt rmak ne Bgd hbak Dbnknf x, 20: 1735-1748.

Agelopoulos, N.G., Keller, M.A., 1994b. Plant-natural enemy association in tritrophic system, Bnsdrha rt addt ka-Chdrhr raoad-Brassicaceae (Cruciferae). III: Collection and identification of plant and frass volatiles. Jnt rmak ne Bgd hbak Dbnknf x, 20: 1955-1967.

Bell, E., Creelman, R.A., Mullet, J.E., 1995. A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Araahcnorhr. Ornbddchmf r ne sgd Nashnmak Abacd x ne Rdhdmbdr ne sgd T mhsdc Rsasdr ne Al drhba, 92: 8675-8679.

Benrey, B., Callejas, A., Rios, L., Oyama, K., Denno, R.F., 1998. The effects of domestication of Ararrhba and Ogardhkt r on the interaction between phytophagous insects and parasitoids. Ahnknf hbak Bnmsrnk 11: 130-140.

Berdegue, M., Reitz, S.R., Trumble, J.T., 1998. Host plant selection and development in Roncnosdra dwhf t a: do mother and offspring know best? Dmsnl nknf ha Dwodrhl dmsakhr ds Aookhbasa, 89: 57-64.

Blaakmeer, A., Geervliet, J.B.F., Van Loon, J.J.A., Posthumus, M.A., Van Beek, T.A., de Groot, A., 1994. Comparative headspace analysis of cabbage plants damaged by two species of Chdrhr caterpillars: consequences for in-flight host location by Bnsdrha parasitoids. Dmsnl nknf ha Dwodrhl dmsakhr ds Aookhbasa, 73: 175-182.

DeMoraes, C.M., Lewis, W.J., Pare, P.W., Alborn, H.T., Tumlinson, J.H., 1998. Herbivore-infested plants selectively attract parasitoids. Nast rd, 393: 570-573.

Dicke, M., 1999. Evolution of induced indirect defense of plants. In: R. Tollrian and C.J. Harevell (eds), Sgd Dbnknfx Amc Dunkt shmm Oe Hmct bhakd Cdedmrdr. Princeton University Press, Princeton, New Jersey, pp.62-88.

Dicke, M., Vet, L.E.M., 1999. Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore. In: H. Olff, V.K. Brown, and R. H. Drent (eds), Hdrahunrdr9Adsv ddm Okamsr Amc Ordcasnrr. Blackwell Science, Oxford, UK, pp.483-520.

Dicke, M., Van Beek, T.A., Ben Dom, N., Van Bokhoven, H. and De Groot, A.E., 1990. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. Jnt rmak ne Bgd hbak Dbnknf x, 16: 381-396.

Dicke, M., Gols, R., Ludeking, D., Posthumus, M.A., 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. Jnt rmak ne Bgd hbak Dbnknf x, 25: 1907-1922.

Du, Y.J., Poppy, G.M., Powell, W., 1996. Relative importance of semiochemicals from first and second trophic levels in host foraging behavior of Aoghct r duh. Jnt rmak ne Bgd hbak Dbnknf x, 22: 1591-1605.

Fidantsef, A.L., Stout, M.J., Thaler, J.S., Duffey, S.S., Bostock, R.M., 1999. Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, Lxbnodrrhbnm drbt kdmst l . Ogxrhnknf hbak amc L nkdbt kar Okams Oasgnknf x, 54: 97-114.

Geervliet, J.B.F., Vet, L.E.M., Dicke, M., 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid Bnsdrha rt addt ka. Entomologia Experimentalis et Applicata, 73: 289-297.

Geervliet, J.B.F., Vet, L.E.M., Dicke, M., 1996. Innate responses of the parasitoids Bnsdrha f knl drasa and B- rt addt ka (Hymenoptera: Braconidae) to volatiles from different plant-herbivore complexes. Jnt rmak ne Hmrdbs Adgauhnr, 9: 525-538.

Geervliet, J.B.F., Posthumus, M.A., Vet, L.E.M., Dicke, M., 1997. Comparative analysis of headspace volatiles from different caterpillar-infested or uninfested food plants of Ohdrhr species. Jnt rmak ne Bgd hbak Dbnknf x, 23: 2935-2954.

Geervliet, J.B.F., Vreugdenhil, A.I., Dicke, M., Vet, L.E.M., 1998. Learning to discriminate between infochemicals from different plant-host complexes by the parasitoids Bnsdrha f knl drasa and B- rt addt ka. Dmsnl nknf ha Dwodrhl dmsakhr ds Aookhbasa, 86: 241-252.

Harvey, J.A., Jervis M.A., Gols, R., Jiang, N., Vet, L.E.M., 1999. Development of the parasitoid, Bnsdrha rt addt ka (Hymenoptera: Braconidae) in Ohdrhr raoad and Ohdrhr ararrhbad (Lepidoptera: Pieridae): Evidence for host regulation. Jnt rmak ne Hmrdbs Ogxrhnknf x, 45: 172-182.

Karban, R., Baldwin, I.T., 1997. Hmct bdc Rdronmrdr sn Hdrahunrx. The University of Chicago Press Ltd, Chicago, USA.

Kessler, A., Baldwin, I.T., 2001. Defensive function of herbivore-induced plant volatile emissions in nature. Rbhdmbd, 291: 2141-2144.

Mattiacci, L., Dicke, M., Posthumus, M.A., 1994. Induction of parasitoid attracting synomone in Brussels sprouts plants by feeding ne Ohdrhr ararrhbad larvae: role of mechanical damage and herbivore elicitor. Jnt rmak ne Bgd hbak Dbnknf x, 20: 2229-2247.

Moran, P.J., Thompson, G.A., 2001. Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways. Okams Ogxrhnknf x, 125: 1074-1085.

Ozawa, R., Arimura, G., Takabayashi, J., Shimoda, T., Nishioka, T., 2000. Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. Okams amc Bdkk Ogxrhnknf x, 41: 391-398.

Renwick, J.A.A., Lopez, K., 1999. Experience-based food consumption by larvae of Pieris rapae: addiction to glucosinolates? Dmsnl nknf ha Dwodrhl dmsakhr ds Aookhbasa, 91: 51-58.

Reymond, P., Weber, H., Damond, M., Farmer, E.E., 2000. Differential gene expression in response to mechanical wounding and insect feeding in Araahcnorhr. Okams Bdkk, 12: 707-719.

Shimoda, T., Dicke, M., 2000. Attraction of a predator to chemical information related to nonprey: when can it be adaptive? Adgauhnrak Dbnknf x, 11: 606-613.

Shiojiri, K., Takabayashi, J., Yano, S., Takafuji, A., 2000. Herbivore-species-specific interactions between crucifer plants and parasitic wasps (Hymenoptera : Braconidae) that are mediated by infochemicals present in areas damaged by herbivores. Aookhdc Dmsnl nknfx amc Znnknfx, 35: 519-524.

Shiojiri, K., Takabayashi, J., Yano, S. & Takafuji, A. 2001. Infochemically mediated tritrophic interaction webs on cabbage plants. Cnot kashmm Dbnknfx 43: 23-29.

Smits, P.H., Van de Vrie, M., Vlak, J.M., 1986. Oviposition of beet armyworm (Lepidoptera: Noctuidae) on greenhouse crops. Dmuhrnml dmsak Dmsnl nknfx, 15: 1189-1191.

Talekar, N.S., Shelton, A.M., 1993. Biology, Ecology, and Management of the Diamondback Moth. Ammt akRduhdv neDmsnl nknfx, 38: 275-301.

Tjallingii, W.F., Hogen Esch, T., 1993. Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. Cgxrhnknfhbak Dmsnl nknfx, 18: 317-328.

Tomczyk, A., Kropczynska, D., 1985. Effects on the Host Plant. In: W. Helle, M. W. Sabelis (eds), Rohcdr l hsdr, sgdhr ahnknfx, mastrak dmdl hdr amc bnmsrnk vol 1A. Elsevier, Amsterdam, The Netherlands, pp 317-329.

Turlings, T.C.J., Bernasconi, M., Bertossa, R., Bigler, F., Caloz, G., Dorn, S., 1998. The induction of volatile emissions in maize by three herbivore species with different feeding habits: Possible consequences for their natural enemies. Ahnknfhbak Bnmsrnk, 11: 122-129.

Turlings, T.C.J., Tumlinson, J.H., Lewis, W.J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. Rbhdmbd 250: 1251-1253

Van Emden, H.F., Eastop, V.F., Hughes, R.D., Way, M.J., 1969. The ecology of L xyt r odrrhbad. Ammt akRduhdv neDmsnl nknfx, 14: 197-270.

Vet, L.E.M., De Jong, A.G., Franchi, E., Papaj, D.R., 1998. The effect of complete versus incomplete information on odour discrimination in a parasitic wasp. Animal Behaviour, 55: 1271-1279.

Vos, M., Hemerik, L., Vet, L.E.M., 1998. Patch exploitation by the parasitoids Bnsdrha rt adbt ka and Bnsdrha fknl drasa in multi-patch environments with different host distributions. Journal of Animal Ecology, 67: 774-783.

Vos, M., Berrocal, S.M., Karamaouna, F., Hemerik, L., Vet, L.E.M., 2001. Plant-mediated indirect effects and the persistence of parasitoid-herbivore communities. Ecology Letters, 4: 38-45.

Walling, L.L., 2000. The myriad plant responses to herbivores. Journal of Plant Growth Regulation, 19: 195-216.

chapter 8

Summarizing discussion:

Arabidopsis and indirect defence

Remco M.P. van Poecke

"No man knows the truth...but, by seeking, men find out, in time, what is better."

Xenophanes

Chapter 8

SUMMARIZING DISCUSSION: *Arabidopsis* AND INDIRECT DEFENCE

1. Why do plants use indirect plant defence?

To defend themselves against herbivorous insects, plants can use both direct and indirect mechanisms (Karban and Baldwin, 1997; Dicke, 1999). Direct defence mechanisms have a direct effect on the herbivore, e.g. by negatively affecting the physiology of the herbivore, using toxic or anti-nutritional compounds, or by interfering with the behaviour of the herbivore, using repelling or deterring compounds (Karban and Baldwin, 1997). Indirect defence mechanisms promote the effectiveness of natural enemies of the herbivore, i.e. predators or parasitoids. One way of promoting the effectiveness of natural enemies is by producing volatiles in response to herbivory that can attract predators or parasitoids (Dicke, 1999). Why plants use indirect defence and how the use of Arabidopsis helps understanding the underlying mechanisms is the topic of this summarizing discussion.

1.1 Indirect defence increases plant fitness

The increased production of volatiles and attraction of carnivores by plants in response to infestation by herbivores is widespread throughout the plant kingdom (Dicke, 1999). Attraction of carnivores can be beneficial for the plant. This is obvious for the attraction of predators as these carnivores kill their herbivorous prey. However, it is less obvious that the attraction of parasitoids benefits a plant. Many parasitoid species are koinobionts, which require their host (the herbivore) to continue feeding and growing after parasitism. Solitary parasitoids, that only lay a single egg per host, usually reduce the feeding and growth of their host compared with unparasitized herbivores. Gregarious parasitoids, which lay clutches of eggs per host, can reduce or increase the feeding and growth of their host, depending on clutch size and host-species (Harvey, 2000). Comparing Arabidopsis plants infested by parasitized or unparasitized P. rapae caterpillars, Van Loon et al. (2000) demonstrated that parasitism of P. rapae by the solitary parasitoid C. rubecula increased plant fitness as measured by seed production. Similarly, parasitism of Spodoptera littoralis by the solitary parasitoid Cotesia marginiventris resulted in an increased seed production by corn plants compared to plants infested by unparasitized caterpillars (Fritzsche-Hoballah and Turlings, 2001).

1.2 Evolution of indirect defences

Although carnivore attraction can benefit the plant, it is unclear how this indirect defence mechanism has evolved. As volatile compounds such as green leaf volatiles and terpenoids have toxic, repellent and antimicrobial effects (Avé et al., 1987; Gershenzon and Croteau, 1991; Croft et al., 1993; Vancanneyt et al., 2001), carnivores might initially have exploited a direct defence mechanism of plants (Dicke, 1999).

However, indirect defences may have an additional benefit for the plant. Herbivores, and especially specialist herbivores that only feed on a few plant species, are renowned for their ability to develop resistance against direct plant defences (Groot and Dicke, 2001). Specialist herbivores may even exploit direct defences to their own benefit during host selection or during the defence against carnivores and most herbivorous insect species are specialists (Schoonhoven et al., 1998). Selection pressure for resistance of herbivores against plant defences increases with the strength of the interaction. This is why it may be more difficult for a herbivore to overcome partial versus complete resistance and inducible, and therefore variable, defences versus constitutive defences (Karban and Baldwin, 1997). Indeed, many direct defences only result in partial resistance against herbivores (Tallamy and Raupp, 1991). Adding an additional trophic level, the carnivores, increases the variability of defences. Therefore, resistance against indirect defence may be more difficult to overcome by herbivores than direct defence.

As the attraction of carnivores can have several benefits for the plant, there is a selective advantage for plants that attract carnivores. Such a selective advantage is the driving force of evolution (Darwin, 1859). However, indirect defences of plants can only have evolved if there is also a benefit for the carnivores. Herbivores are small components of a complex environment and most herbivore species have evolved to be inconspicous to their natural enemies. Therefore herbivore-derived stimuli often have a low detectability. Plant-derived stimuli can be much easier to detect, as plants have a much larger biomass compared to herbivores and additionally they benefit from the attraction of the natural enemies of herbivores. Besides being detectable, information for the carnivores has to be reliable. Especially when a plant is a host for many herbivore species it is important for the plant to emit volatile blends with a certain herbivore-specificity. After all, most parasitoid and several predator species are specialized on only one or a few herbivore species. A general volatile blend emitted by plants upon herbivory that provides no information on the herbivore species attacking the plant is of little help for these specialist carnivores. As plants can emit different volatile blends when infested by different herbivore species (discussed in more detail in the following sections of this chapter), it is likely that coevolution between carnivores and plants has stimulated herbivory-induced volatile production by plants, resulting in what we now call indirect defence (Vet and Dicke, 1992; Dicke and Vet, 1999; Dicke, 1999; Sabelis et al., 1999).

1.3 Direct versus indirect defences

Direct and indirect defence can act synergistically or antagonistically. Partial direct defence often results in a lower development rate of the herbivore compared to susceptible plants. This extends the time available for carnivores to find their prey (Dicke, 1999). On the other hand, toxins that are produced as direct defence compounds by plants can also negatively affect the carnivore (**chapter 2**). They can be sequestered by specialist herbivores, rendering the herbivores toxic to the carnivores (Barbosa et al., 1986; Krischik et al., 1988). In this light, it is interesting that in tobacco wounding results in elevated nicotine levels that is correlated to the elevated levels of the plant hormone jasmonic acid (JA) after wounding (Baldwin et al., 1997). However, feeding by the specialized, nicotine-tolerant herbivore Manduca sexta amplified the wound-induced increases of JA but not of nicotine (McCloud and Baldwin, 1997; Kahl et al., 2000). As M. sexta-infested tobacco plants emit herbivory-induced volatiles, it is possible that this represents a response where plants enforce their indirect defence at the expense of non-functional direct defence mechanisms that can harm carnivores. This also illustrates that direct and indirect defence mechanisms can be uncoupled (Kahl et al., 2000).

1.4 The complexity of inducible defences

In short, plants can orchestrate complex responses to herbivores, by differentially inducing direct and indirect defences that also depend on the herbivore species. Information on the mechanisms behind these complex responses is limited. To get a better understanding of this, we studied indirect defences in the model plant Arabidopsis thaliana.

2. *Arabidopsis* as a model plant for studying indirect defence

For a plant species to be suitable as a model plant, it has to meet certain requirements:

- The model plant has to possess the trait of interest.
- The trait of interest should be similar in the model plant and the other plant species it represents.
- It should be easier to study the trait in the model plant than in other plant species.
- Other, related traits are studied in the same model plant species.

I will discuss these requirements with respect to Arabidopsis as a model plant for studying indirect defence, starting with the last two.

2.1 It is easier to study the trait in the model plant than in other plant species

Several properties have made Arabidopsis the model plant for molecular genetic studies. These include a short life cycle, the prolific production of seeds, a small genome that is completely sequenced, and the ease of genome manipulation

(Meinke et al., 1998). As a result, an astonishing number of different mutant and transgenic plants are now available, including those with altered or inhibited signal-transduction pathways. Use of these mutant and transgenic plants makes it possible to study signal transduction in ways that could not have been achieved using exogenous application of elicitors or inhibitors. This makes Arabidopsis thaliana a unique plant species in contemporary science.

2.2 Other, related traits are studied in the same model plant species

As the use of Arabidopsis provides unique possibilities for studying signal-transduction pathways, the knowledge on signal transduction in plant defence in Arabidopsis is rapidly growing. This is especially true for studies on defences against pathogens (Glazebrook, 2001), whereas studies on defences against herbivorous insects using Arabidopsis have recently been initiated (McConn et al., 1997; Mauricio, 1998; Reymond et al. 2000; Stotz et al., 2000; Van Loon et al., 2000; this thesis).

2.3 The model plant has to possess the trait you want to study

To study indirect defences in Arabidopsis, one first needs to demonstrate that Arabidopsis becomes more attractive to carnivores when infested with herbivores. Using a wind tunnel set up female Cotesia rubecula wasps were offered a choice between undamaged Arabidopsis plants and Arabidopsis plants infested with Pieris rapae caterpillars. The wasps showed a strong preference for herbivore-infested plants. Moreover, a single Arabidopsis plant infested with a single first instar P. rapae larva became more attractive as the infestation duration increased (**chapter 3**). Chemical analysis of the volatile blend showed that P. rapae feeding resulted in the emission of volatiles that were not or in lower amounts emitted by undamaged plants. Especially methyl salicylate (MeSA), the homoterpene (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), 1-penten-3-ol, and to a lesser extent the monoterpene myrcene, the green leaf volatiles (Z)-3-hexen-1-ol and 1-hexanol, the two nitriles 5-(methylthio)-pentanenitrile and 6,7-dithiooctanenitrile, and dimethyltrisulfide are emitted in larger amounts by P. rapae-infested plants (**chapters 3 and 5**). These compounds are commonly found in the herbivory-induced volatile blend of different plant species (McCall et al., 1994; Geervliet et al., 1997; Turlings et al., 1998; Dicke et al., 1999; Gols et al., 1999).

2.4 The trait of interest should be similar in the model plant and the other plant species it represents

In the last decade, much progress has been made in the study on indirect plant defences by herbivory-induced volatile production. We investigated whether some of the most important findings are comparable in our model system.

2.4.1 Herbivore damage versus mechanical damage

For several plant species it has been demonstrated that the volatile blends emitted by mechanically damaged and herbivore-infested plants differ (see **chapter 2**).

Some plant species, such as Lima bean and corn, emit volatiles upon herbivory that are hardly or not emitted by undamaged or mechanically damaged plants (Dicke et al., 1990b; Turlings et al., 1990). Other plant species, such as Brussels sprouts and cotton, emit a volatile blend in response to herbivory that is qualitatively similar to the blend from undamaged or mechanically damaged plants, but do so in larger amounts (Mattiacci et al., 1994; Rose et al., 1996). **Chapter 3** shows that the volatile blends of mechanically damaged plants and P. rapae-infested plants differ. Similar to Brussels sprouts, which just as Arabidopsis belongs to the Brassicaceae plant family, caterpillar-infested Arabidopsis emit a volatile blend that is qualitatively similar to the blend emitted by mechanically damaged Arabidopsis. Most of the volatiles present in the blend of P. rapae-infested Arabidopsis are also found in the blend of mechanically damaged plants, with the exception of a few compounds such as myrcene and the two nitriles. Methyl salicylate and TMTT were emitted in larger amounts by P. rapae-infested plants, whereas mechanically damaged plants emitted larger amounts of green leaf volatiles.

Carnivores, including C. rubecula, can distinguish between mechanically damaged and herbivore-infested plants (Turlings et al., 1990; Geervliet et al., 1994; Mattiacci et al., 1994; Rose et al., 1998). This is also true with respect to volatiles from Arabidopsis, as C. rubecula preferred P. rapae-infested Arabidopsis to mechanically damaged plants (**chapters 3 and 6**).

2.4.2 Herbivore-derived elicitors

As mechanical damage and herbivore damage induce distinct responses, this indicates that, similar to other plant species, herbivore-derived elicitors are involved in herbivory-induced volatile production and parasitoid attraction by Arabidopsis. However, as it is very difficult to mimic the mechanical part of herbivory (Baldwin, 1990), comparison between mechanically damaged and herbivore-infested plants is not conclusive. Simulating herbivory by treating mechanically damaged plants with caterpillar regurgitant has provided more compelling evidence. Regurgitant treatment resulted in the emission of a volatile blend that closely resembles the blend of herbivore-infested plants in for example corn, Brussels sprouts and tobacco (Turlings et al., 1990; Mattiacci et al., 1994; Halitschke 2001). Several carnivore species were more attracted to mechanically damaged plants treated with regurgitant than to plants that were only mechanically damaged (Turlings et al., 1990; Mattiacci et al., 1994; Takabayashi et al. 1995; Rose et al., 1998). Similarly, mechanically-damaged Arabidopsis plants treated with Pieris regurgitant were more attractive than Arabidopsis plants that were only mechanically damaged (**chapter 6**).

2.4.3 The response of C. rubecula to Arabidopsis infested by different herbivore species

The blend emitted by herbivore-infested plants does not only differ between different plant species, but may also depend on the herbivore species infesting the plant (Takabayashi et al., 1991; Blaakmeer et al., 1994; Geervliet et al., 1997;

Turlings et al., 1998). This is partly due to the fact that different herbivore species cause different types of damage. Feeding by leaf-chewing insects such as caterpillars results in more tissue-disruption or wounding of the plant than feeding by phloem-feeders such as aphids. Besides this, a difference in herbivore-derived elicitors is likely to be involved (Walling, 2000).

How the plant responds to feeding by different herbivore species depends on the plant species. Some plant species emit blends that are qualitatively different (Turlings et al., 1998; Du et al. 1998), other plant species emit blends that are quantitatively different (Takabayashi et al., 1991). Brassicaceous plants such as Brussels sprouts, and white, red, and green cabbage emit volatile blends with minor quantitative differences when infested by different herbivore species (Blaakmeer et al., 1994; Geervliet et al., 1997). When these plant varieties were infested by different lepidopteran herbivore species, C. rubecula did not distinguish between plants infested with its host P. rapae and plants infested with non-host caterpillars such as Plutella xylostella (Agelopoulos and Keller, 1994; Geervliet et al., 1996). However, Cotesia rubecula preferred host-infested cabbage to cabbage infested with a non-host non-lepidopteran herbivore (the snail Helix aspera; Agelopoulos and Keller, 1994). The response of carnivores towards cabbage plants infested with different herbivore species also depends on the carnivore species. The parasitoid Cotesia plutellae did distinguish between cabbage plants infested by host and non-host infested caterpillars, preferring the first (Shiojiri et al., 2000). After learning, C. glomerata could distinguish between Brussels sprouts plant infested by P. rapae and plants infested by a more suitable host, P. brassicae, preferring the latter (Geervliet et al., 1998).

We demonstrated that C. rubecula responds in a similar way to Arabidopsis plants infested by host and non-host lepidopteran species as it does to cabbage plants (**chapter 7**). The parasitoid did not distinguish between Arabidopsis infested by P. rapae and Arabidopsis infested with caterpillars of Plutella xylostella or Spodoptera exigua. Additionally, we demonstrated that C. rubecula does distinguish between volatiles emitted by host-infested Arabidopsis and Arabidopsis infested with non-lepidopteran non-hosts, i.e. Tetranychus urticae spider mites and Myzus persicae aphids, preferring host-infested Arabidopsis.

2.4.4 Jasmonic acid and the induction of volatile emission

The herbivory-induced emission of volatiles is not a passive process but results from an increased production of the volatiles by the plants. This has been shown for several plant species. Evidence comes from the fact that:

- Herbivore-derived elicitors are involved in the emission of herbivore-induced volatiles in a range of plant species (Mattiacci et al., 1995; Alborn et al., 1997; Halitschke et al., 2001).
- The activity of an enzyme involved in terpene biosynthesis was induced by herbivore feeding in cucumber, Lima bean and corn (Bouwmeester et al., 1999; Degenhardt & Gershenzon 2000).

- Pulse-labeling studies showed an increased incorporation of radioactive ^{13}C when mechanically damaged cotton plants were treated with regurgitant (Pare and Tumlinson, 1997).

As herbivory results in the increased production of volatiles, signal transduction from herbivory to volatile production is required. Evidence has been accumulating that the plant hormone jasmonic acid (JA) is involved in this signal transduction:

- Herbivory induces an increase of endogenous JA levels in broad bean, tobacco and Arabidopsis (Blechert et al., 1995; McCloud and Baldwin, 1997; Stotz et al., 2002).
- In Lima bean, gerbera, tobacco and corn exogenous application of JA, methyl jasmonate (MeJA) or JA-inducing compounds like cellulysin induces a similar, although not identical volatile blend compared to herbivore feeding (Piel et al., 1997; Dicke et al., 1999; Gols et al., 1999; Kessler and Baldwin, 2001).
- Inhibition of the octadecanoid pathway, that produces JA, blocked the induced emission of volatiles by cellulysin in Lima bean (Piel et al., 1997).
- Exogenous application of JA to plants induces carnivore attraction to Lima bean and gerbera plants in the laboratory (Dicke et al., 1999; Gols et al. 1999) and to tomato plants in the field (Thaler, 1999).

In **chapters 4 and 5** we demonstrated that exogenous application of JA to Arabidopsis results in increased emission of volatiles, such as terpenoids and MeSA, and that these volatiles attract C. rubecula.

3. Novel aspects of herbivory-induced volatiles: the benefits of a molecular genetic approach

The value of a molecular genetic approach in studies on insect-plant interactions has been pointed out by several scientists (Mitchell-Olds et al., 1998; Kessler and Baldwin, 2002, Dicke et al., 2002). The use of Arabidopsis thaliana, the model plant in contemporary science for molecular genetic studies, provides an ideal model system to study signal transduction involved in indirect defences against herbivores and how this is related to signal transduction in direct defences. The following section discusses the results we obtained by using Arabidopsis in a molecular genetic approach to unravel signal-transduction in indirect defence (Table 1).

3.1 Synergism between JA and SA in herbivory-induced volatile production

In Lima bean, the volatile blends emitted by JA-treated and caterpillar infested plants closely resembled each other (Ozawa et al., 2000). Infestation with another herbivore species, spider mites, induced a volatile blend that was different from the JA-induced blend. The major differences were that spider mite feeding induced the emission of MeSA and TMTT, whereas JA treatment did not (Dicke et al., 1999). Successive treatment of Lima bean with JA and MeSA did result in the emission of TMTT (Ozawa et al., 2000). The predatory mite Phytoseiulus persimilis, that feeds

Table 1. Mutant and transgenic plants that were studied in this thesis

mutant/ transgenic	**phenotype**	**reference**
Nbi H	Does not accumulate SA due to expression of to the expression of the bacterial salicylate hydroxylase gene *Nbi H*	Delaney *fubm,* 1994
S-12	Does not show wound-induced accumulation of JA due to cosuppresion of *MP* X2	Bell *fubm,* 1995
ops2.2	non-expresser of pathogenesis related (PR) genes	Cao *fubm,* 1994
jbs2.2	jasmonic acid resistant	Staswick *fubm,* 1992

on spider mites, preferred spider-mite-infested plants to JA-treated or caterpillar-infested plants. This suggests an important role for SA in the signal transduction of induced attraction of predatory mites by spider mite-infested Lima bean (Dicke et al., 1999; Shimoda and Dicke, 2000). These data indicate that different herbivore species induce different plant hormones, with caterpillar feeding inducing JA-dependent responses and spider mite-feeding inducing both JA- and SA-dependent responses (Walling, 2000; Ozawa et al. 2000).

In Arabidopsis, feeding by the chewing herbivore P. rapae resulted in the attraction of C. rubecula. Feeding by cell-sucking spider mites also resulted in the attraction of C. rubecula, but the wasps preferred P. rapae-infested to spider mite infested Arabidopsis. Infestation with a phloem-feeding aphid did not result in the attraction of C. rubecula (**chapter** 7). Contrary to the results obtained with S. exigua Lima bean, SA is involved in caterpillar-induced volatile emissions by Arabidopsis. Transgenic NahG plants that cannot accumulate SA (Delaney et al., 1994) did not emit MeSA and TMTT when infested by P. rapae in contrast to wild-type Arabidopsis, demonstrating a signalling role for SA in caterpillar-induced volatile production (**chapter 5**). Cotesia rubecula preferred P. rapae-infested wild-type plants to P. rapae-infested NahG plants (**chapter** 4), indicating that MeSA and TMTT are important parasitoid attractants. However, P. rapae-infested NahG plants attracted more wasps than uninfested NahG plants, demonstrating an additional role for other plant hormones such as JA. Indeed, treatment of Arabidopsis with only SA did not result in the attraction of C. rubecula (**chapter** 4).

These differences might be due to both differences in herbivore derived elicitors and different types of feeding (Walling, 2000), with chewing insects such as caterpillars (Baldwin, 1990) mainly inducing JA, phloem-feeding insects such as aphids (Tjallingii and Hogen-Esch, 1993) mainly inducing SA and cell-piercing herbivores such as spider mites (Tomczyk and Kropczyńska, 1985) inducing more equal levels of both JA and SA. Indeed, aphid feeding on tomato resulted in a strong induction of SA-inducible defences, but had only a weak effect on JA-inducible defences (Moran and Thompson, 2001).

3.2 Cross-talk between the octadecanoid and salicylic acid pathway

In contrast to the synergism between JA and SA in herbivory-induced volatile production, several publications reported negative cross-talk between the octadecanoid and salicylic acid pathway (Felton and Korth, 2000; Pieterse *et al.*, 2001a). This may lead to situations where induced defence against one attacker results in a reduced defence against another. Tobacco plants infested with tobacco mosaic virus (TMV), resulting in increased SA levels, were more susceptible to *Manduca sexta* caterpillars (Preston *et al.*, 1999). Transgenic tobacco plants with reduced SA levels were more susceptible to TMV but showed increased resistance against *Heliothis virescens* caterpillars (Felton *et al.*, 1999). The octadecanoid pathway is known to be important for direct defences against herbivores (**chapter 2**) and both the octadecanoid and salicylic acid pathway are involved in defences against pathogens. For example, systemic acquired resistance (SAR) of *Arabidopsis* against pathogens induced by biotrophic pathogens requires SA (Dong, 2001), and induced systemic resistance (ISR) of *Arabidopsis* against pathogens induced by rhizobacteria requires JA (Pieterse *et al.*, 2001b). The induction of both ISR and SAR resulted in an additive effect in the resistance of *Arabidopsis* against *Pseudomonas syringae* pv. tomato. Together, these findings demonstrate that the octadecanoid and salicylic acid pathways can act both antagonistically and synergistically in plant defences (Van Wees *et al.*, 2000).

3.3 The role of OPDA in herbivory-induced volatile production

There are indications that, besides JA, another compound from the same biosynthetic pathway (the octadecanoid pathway) is involved in signal transduction that leads to herbivory-induced volatile production. This compound is 12-oxo-phytodienoic acid (OPDA), an intermediate in the octadecanoid pathway that leads to JA synthesis. Treatment of *Arabidopsis* with SA is known to increase OPDA levels without a subsequent increase of JA. To be processed to JA, OPDA needs to be transported from the chloroplast, where it is synthesized, to the cytosol. The transport through the chloroplast membrane is most likely inhibited by SA (Laudert and Weiler, 1998). Thus, herbivore feeding, inducing both the octadecanoid and salicylic acid pathway, probably also induces increased OPDA levels. Exogenous application of OPDA to Lima bean plants resulted in increased emission of TMTT, a volatile that is not induced by JA-treatment (Koch *et al.*, 1999) and inhibition of SA accumulation in *Arabidopsis* inhibited herbivory-induced emission of TMTT (**chapter 5**). These results indicate that SA acts as a regulator of OPDA and JA accumulation, stimulating OPDA and inhibiting JA accumulation, and as such as a modulator of herbivory-induced volatile production. JA is possibly involved in the accumulation of OPDA in *Arabidopsis* as JA treatment induced MeSA emission and therefore likely SA levels. The inhibition of JA accumulation is not complete in *Arabidopsis* and might be dose dependent. This is indicated by the fact that spider mite feeding on Lima bean induces the emission of both JA- and OPDA-dependent volatiles, probably through activation of both JA- and SA-dependent pathways (Dicke *et al.*, 1999; Ozawa *et al.*, 2000). Moreover, treatment of Lima bean with the pathogen-derived elicitor alamethicin strongly induces the production of SA and only induces the emission of OPDA-dependent volatiles (Engelberth *et al.*, 2001). The alamethicin-induced Lima bean volatiles attracted the carnivorous mite P. persimilis (**chapter 2**).

3.4 Increased sensitivity to JA

The JA-dependent induction of volatile emissions might not only be the result of increased endogenous levels of JA but also of increased sensitivity to JA. This is supported by studies on transgenic S-12 Arabidopsis plants that do not show wound-induced accumulation of JA, although basal levels of JA are maintained (Bell et al., 1995). When infested by P. rapae S-12 plants emit a volatile blend that is qualitatively similar to that of P. rapae-infested wild-type plants (**chapter 5**). This is in contrast to our expectation that the emission of JA-dependent volatiles such as MeSA and terpenoids, especially myrcene, would be inhibited. A similar observation was made by Pieterse et al. (2001b), who showed that induced systemic resistance against pathogens, although dependent on JA, was unaffected in S-12 plants. Such an increased sensitivity might also explain why expression of AOS, HPL, PAL1, and TPS10 (Table 2) was similar in wild-type and S-12 plants after 24 h of P. rapae feeding (**chapter 5**), even though AOS is known to be induced by JA in Arabidopsis (Laudert and Weiler, 1998) and PAL genes are known to be induced by JA in other plant species (Creelman et al., 1992; Gundlach et al., 1992).

Table 2. Genes involved in induced volatile production that were studied in this thesis

Gene	Enzyme	Function	Reference
MPX2	lipoxygenase	First biosynthetic enzyme of the octadecanoid pathway, involved in wound-inducible accumulation of JA.	Bell *fubm,* 1995
BPT	allene oxide synthase	Enzyme downstream of LOX directing LOX products toward the production of OPDA and JA.	Laudert and Weiler, 1998
HPM	hydroperoxide lyase	Enzyme downstream of LOX directing LOX products toward the production of green leaf volatiles.	Bate *fubm,* 1998
PBM2	phenylalanine ammonia-lyase	Enzyme involved in the production of SA and MeSA	Mauch-Mani and Slusarenko, 1996
UPT20	myrcene/(*F*)-*b*-ocimene synthase	Enzyme involved in the production of myrcene and small amounts of (*F*)-*b*-ocimene	Bohlmann *fubm,* 2000

Surprisingly, although the quality of the volatile blend was very similar between P. rapae-infested wild-type and S-12 plants, meaning that the ratios of the different compounds in the blend were similar, the quantity was not. Infested S-12 plants emitted lower amounts of volatiles than infested wild-type plants (**chapter 5**), including compounds that are constitutively emitted in wild-type plants (**chapter 3**). Jasmonic acid is reported to be involved in the development of stomata

(Bougouin and Horton, 1992). Therefore cosuppression of LOX2 might influence stomatal development. Indeed, preliminary data show that the leaf surface of S-12 plants contains two times less stomata per surface unit compared to wild-type plants. Additionally, S-12 plants lack trichomes, reducing the total leaf surface. Both these phenotypic differences indicate that volatiles might be less easily emitted by S-12 plants.

3.5 Signalling downstream of jasmonic and salicylic acid

Plant hormones are not the only components of signal transduction pathways (**chapter 2**). First of all, the stimuli have to be perceived. Stimulation by herbivory arises from mechanical wounding and herbivore-derived elicitors, such as β-glucosidase found in the regurgitant of P. brassicae (Mattiacci et al., 1995), glucose oxidase found in the regurgitant of Helicoverpa zea (Musser et al., 2002) and fatty-acid amino acid conjugates found in the regurgitant of M. sexta, M. quinquemaculata and S. exigua (Alborn et al., 1997; Halitschke et al., 2001). Among the first events following perception of the stimuli are ion-fluxes, calcium signalling, reversible protein phoshorylation and production of reactive oxygen species (De Bruxelles and Roberts, 2001; León et al., 2001). Some of these first events likely trigger the increase of hormone levels or hormone susceptibility. For example, activation of phospholipases is necessary for the release of linolenic acid from the chloroplast membrane. Linolenic acid is a substrate for lipoxygenases, and as such required for production of OPDA and JA through the octadecanoid pathway (Schaller, 2001). Wound-inducible phospholipases are activated by calcium and modulated by reversible phosphorylation (León et al., 2001).

Subsequently, activation of hormonal pathways has to result in gene-expression, which, in the case of herbivory-induced volatiles, ultimately leads to the induced production and emission of volatiles. These events downstream of plant hormones such as JA and SA involve reversible phosphorylation and the activation of transcription factors (Glazebrook, 2001; León et al., 2001).

An important factor in the regulation of signal transduction downstream of SA and JA is NPR1. Mutations in the NPR1 gene results in a loss of SAR and ISR in Arabidopsis that could not be restored by JA or SA application which indicates that NPR1 acts downstream of JA and SA (Cao et al., 1994; Pieterse et al., 1998). The NPR1 protein regulates gene-expression by binding to transcription factors belonging to the TGA family. These transcription factors bind to TGA motifs present in the promoters of genes such as PR1 (Zhang et al., 1999). Another factor that acts downstream of JA is JAR1. Although the function of JAR1 is not yet known, mutations in the JAR1 gene resulted in a reduced sensitivity to JA, in a loss of ISR and in increased susceptibility to the soil fungus Pythium irregulare (Pieterse et al., 1998; Staswick et al., 1998).

Using npr1-1 and jar1-1 mutant Arabidopsis plants we demonstrated that P. rapae-induced attraction of C. rubecula is unaffected by these mutations (**chapter 6**). This indicates/shows/demonstrates that the signal transduction required for indirect defence against herbivores differs from the signal transduction required for ISR and SAR against pathogens. As ISR and SAR result in resistance against pathogens and as defences against pathogens and herbivores can be very different, this may not seem very surprising. However, as JA and SA play key roles in defences against both pathogens and herbivores, this indicates that the signal transduction pathways of these defences overlap at least partially. Indeed, the defences induced in tomato by the pathogen P. syringae pv. tomato also result in an increased resistance against the herbivore H. zea and vice versa (Stout et al., 1999). Recently, it was demonstrated that npr1-1 mutants showed enhanced resistance against the generalist caterpillar Spodoptera littoralis (Stotz et al., 2002). Whether this means that in Arabidopsis direct and indirect defence mechanisms against caterpillars are induced through separate signal-transduction pathways, or that generalist caterpillars such as S. littoralis induce different signal-transduction pathways than specialists such as P. rapae remains to be investigated. Additionally, induced expression of PR-genes by aphid infestation was reduced in the npr1-1 mutant, although this did not affect aphid reproduction (Moran and Thompson, 2001). Clearly, the pathways involved in defence against insects and pathogens can overlap also downstream of JA and SA.

Some inducible defences of Arabidopsis against pathogens are JA-dependent but not dependent on JAR1 and NPR1 and others are SA-dependent but not NPR1-dependent (for discussion see **chapter 6**). It would be interesting to investigate whether mutants affected in these NPR1- and JAR1-independent pathways affect indirect defences of Arabidopsis against herbivores.

3.6 Signal transduction in induced defences of Arabidpsis: future perspectives

Although the work described in this thesis addressed some aspects of signal-transduction in indirect defences, naturally many questions remain and new questions arose. I will briefly touch on some of these.

Although there is circumstantial evidence for an important role of OPDA in herbivory-induced volatile production, more studies are required for a more solid conclusion. Recently, a mutant Arabidopsis plant was described that does not convert OPDA to JA. In contrast to plants that cannot accumulate both OPDA and JA (McConn et al., 1997), this mutant retains resistance against herbivory by fungal gnats (Stintzi et al., 2001). Resistance of Arabidopsis against the fungal pathogen Alternaria brassicicola, that was demonstrated to be octadecanoid-dependent but NPR1-independent (Thomma et al., 1998), was unaffected in this mutant (Stintzi et al., 2001). It would be interesting to study whether indirect defence is affected in this mutant.

Another plant hormone that may play a role in herbivory-induced volatile production is ethylene. Spider mite-infestation induced the production of ethylene (Arimura et al., 2002). Exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) to Lima bean plants enhanced the JA-induced emission of some volatiles. Moreover, a combination of JA and ACC treatment induced the attraction of the predatory mite P. persimilis whereas treatment of either JA or ACC at similar concentrations as used in the combination did not (Horiuchi et al., 2001). Herbivory-induced ethylene production in tobacco inhibited nicotine accumulation but not the emission of volatiles (Kahl et al., 2000). In tomato, the induction of proteinase inhibitor (PIN) genes involved in the direct defence against herbivores by JA requires ethylene (O'Donnell et al., 1996). But blocking ethylene signalling did not affect resistance of Arabidopsis against P. xylostella caterpillars and resulted in increased resistance against S. littoralis caterpillars (Stotz et al., 2000). No difference in wound-induced gene-expression was found in an ethylene-sensitive Arabidopsis mutant compared to wild-type plants (Reymond et al., 2000). However both JA and ethylene are required for ISR (Pieterse et al., 1998). In short, the role of ethylene in plant defences is unclear. Possibly ethylene can inhibit direct but stimulate indirect defence mechanisms.

The induced emission of volatiles by herbivory is not limited to the damaged parts of the plant. Undamaged parts of the plant also show and increased production and emission of volatiles (Turlings and Tumlinson, 1992; Dicke et al., 1990a, 1993; Potting et al., 1995; Pare and Tumlinson, 1998). Thus, a long distance signal (systemic signal) in the plant is required. The possible role of JA and SA as systemic signals is still under debate (Ryals et al., 1994; De Bruxelles and Roberts, 2001; Métraux, 2001; Kessler and Baldwin, 2002). Probably the best-studied candidate for systemic signalling is systemin, which has been found in several solanaceous plant species (Ryan, 2000 ref in de brux). However, no systemin analogue has been found in species from other plant families, including Arabidopsis. Thus, the quest for the systemic signals is far from over.

Possibly the most intriguing question is how plants can orchestrate the broad spectrum of different defence mechanisms that depend on herbivore and pathogen species and can enhance or inhibit each other. For example, differences may be due to the induction of different levels of plant hormones, different sensitivity to these plant hormones, different groups of plant hormones, different cell compartments, and different temporal patterns. Besides this, other factors like active oxygen species, oligogalacturonides, electrical currents, ion-fluxes, hydraulic waves, and protein phosphorylation are likely play a role (León et al., 2001; Schaller and Weiler, 2002; **chapter 2**). Arabidopsis has already proven to be a useful model plant for studying direct defences against pathogens and herbivores. The work described in this thesis demonstrates that Arabidopsis is an excellent model plant for studying indirect defences and will be very useful in enlightening the complex interactions of signalling in defence mechanisms by plants.

9. References

Agelopoulos, N.G., Keller, M.A., 1994. Plant-natural enemy association in the tritrophic system Cotesia rubecula-Pieris rapae-Brassicaceae (Cruciferae): II. Preference of C. rubecula for landing and searching. J. Chem. Ecol. 20: 1735-1748.

Arimura, G.I., Ozawa, R., Nishioka, T., Boland, W., Koch, T., Kühnemann, F., Takabayashi, J. (2002). Herbivore-induced volatiles induce the emission of ethylene in neighbouring lima bean plants. Plant J. 29: 87-98.

Agelopoulos, N.G., Keller, M.A., 1994. Plant-natural enemy association in the tritrophic system Cotesia rubecula-Pieris rapae-Brassicaceae (Cruciferae): II. Preference of C. rubecula for landing and searching. J. Chem. Ecol. 20: 1735-1748.

Arimura, G.I., Ozawa, R., Nishioka, T., Boland, W., Koch, T., Kühnemann, F., Takabayashi, J. (2002). Herbivore-induced volatiles induce the emission of ethylene in neighbouring lima bean plants. Plant J. 29: 87-98.

Alborn, T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H. and Tumlinson, J.H. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. Science 276: 945-949.

Avé, D.A., Gregory, P. and Tingey, W.M. (1987). Aphid repellent sesquiterpenes in glandular trichomes of Solanum berthaultii and S. tuberosum. Entomol. Exp. Appl. 44: 131-138.

Baldwin, I.T. (1990). Herbivory Simulations in Ecological Research. Trends Ecol. Evol. 5: 91-93.

Baldwin, I.T., Zhang, Z.P., Diab, N., Ohnmeiss, T.E., McCloud, E.S., Lynds, G.Y. and Schmelz, E.A. (1997). Quantification, correlations and manipulations of wound-induced changes in jasmonic acid and nicotine in Nicotiana sylvestris. Planta 201: 397-404.

Barbosa, P., Saunders, J.A., Kemper, J., Trumbule, R., Olechno, J., Martinat, P. (1986). Plant allelochemicals and insect parasitoids. Effects of nicotine on Cotesia congregata (Say) (Hymenoptera: Braconidae) and Hyposoter annulipes (Cresson) (Hymenoptera: Ichneumonidae). J. Chem. Ecol. 12:1319-1328.

Bate, N.J., Sivasankar, S., Moxon, C., Riley, J.M.C., Thompson, J.E. and Rothstein, S.J. (1998). Molecular characterization of an Arabidopsis gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible. Plant Physiology 117: 1393-1400.

Bell, E., Creelman, R.A. and Mullet, J.E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc. Natl. Acad. Sci. USA 92: 8675-8679.

Blaakmeer, A., Geervliet, J.B.F., Van Loon, J.J.A., Posthumus, M.A., Van Beek, T.A., De Groot, A.E. (1994). Comparative headspace analysis of cabbage plants damaged by two species of Pieris caterpillars: consequences for in-flight host location by Cotesia parasitoids. Entomol. Exp. Appl. 73: 175-182.

Blechert, S., Brodschelm, W., Holder, S., Kannerer, L., Kutcham, T., Mueller, M.J., Xia, Z.Q. and Zenk, M.H. (1995). The octadecanoic pathway: signal molecules for the regulation of secondary pathways. Proc. Natl. Acad. Sci. USA 92: 4099-4105.

Bohlmann, J., Martin, D., Oldham, N.J. and Gershenzon, J. (2000). Terpenoid secondary metabolism in Arabidopsis thaliana: cDNA cloning, characterization, and functional expression of a myrcene/(E)-beta-ocimene synthase. Arch. Biochem. Biophys. 375: 261-269.

Bourgouin, N.C., Horton, R.F. (1992) Heterophylly in amphibious Ranunculi: the effects of abscisic acid and methyl jasmonate. In Current Plant Science and Biotechnology in Agriculture Vol 13: Progress in plant growth regulation: proceedings of the 14th international conference on plant growth substances held in Amsterdam on 21-26 July 1991. (Karssen, C.M., Loon, L.C. van, Vreugdenhil, D.) Dordrecht, the Netherlands: Kluwer Academic Publishers, pp. 386-392.

Bouwmeester, H.J., Verstappen, F.W.A., Posthumus, M.A. and Dicke, M. (1999). Spider mite-induced (3S)-(E)-nerolidol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. Plant Physiol. 121: 173-180.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X.N. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6: 1583-1592.

Creelman, R.A., Tierney, M.L. and Mullet, J.E. (1992). Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl. Acad. Sci. U. S. A. 89: 4938-4941.

Croft, K.P.C., Juttner, F. and Slusarenko, A.J. (1993). Volatile products of the lipoxygenase pathway evolved from Phaseolus vulgaris (L.) leaves inoculated with Pseudomonas syringae pv. phaseolicola. Plant Physiol. 101: 13-24.

Darwin, C. (1859). On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. John Murray, London, UK.

De Bruxelles, G.L. and Roberts, M.R. (2001). Signals regulating multiple responses to wounding and herbivores. Crit. Rev. Plant Sci. 20: 621-621.

Degenhardt J. and Gershenzon, J. (2002). Demonstration and characterization of (*E*)-nerolidol synthase from maize: a herbivore-inducible terpene synthase participating in (3*E*)-4,8-dimethyl-1,3,7-nonatriene biosynthesis. *Planta* 210: 815-822.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut Rella, M., Kessmann, H., Ward, E. et al. (1994). A central role of salicylic acid in plant disease resistance. *Science* 266: 1247-1250.

Dicke, M. (1999). Evolution of induced indirect defense of plants. In *The Ecology And Evolution Of Inducible Defenses*, (R. Tollrian and C.D. Harvell, eds.). Princeton University Press, Princeton, New Jersey, pp. 62-88

Dicke, M., Baarlen, P.v., Wessels, R., Dijkman, H. and Van Baarlen, P. (1993). Herbivory induces systemic production of plant volatiles that attract predators of the herbivore: extraction of endogenous elicitor. *J. Chem. Ecol.* 19: 581-599.

Dicke, M., Sabelis, M. W., Takabayashi, J., Bruin, J. & Posthumus, M. A. (1990a). Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control. *J. Chem. Ecol.* 16: 3091-3118.

Dicke, M., Van Beek, T.A., Ben Dom, N., Van Bokhoven, H. and De Groot, A.E. (1990b). Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16: 381-396.

Dicke, M., Gols, R., Ludeking, D. and Posthumus, M.A. (1999). Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25: 1907-1922.

Dicke, M. and Vet, L.E.M. (1999). Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore. In *Herbivores Between Plants And Predators*, (H. Olff, V.K. Brown, R.H. Drent, eds.). Princeton University Press, Princeton, New Jersey, pp. 483-520

Du, Y. J., Poppy, G. M., Powell, W., Pickett, J. A., Wadhams, L. J., Woodcock, C. M. (1998). Identification of semiochemicals released during aphid feeding that attract parasitoid *Aphidius ervi*. *J. Chem. Ecol.* 24: 1355-1368.

Dong, X.N. (2001). Genetic dissection of systemic acquired resistance. *Curr. Opin. Plant Biol.* 4: 309-314.

Engelberth, J., Koch, T., Schuler, G., Bachmann, N., Rechtenbach, J. and Boland, W. (2001). Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean. *Plant Physiol.* 125: 369-377.

Felton, G.W. and Korth, K.L. (2000). Trade-offs between pathogen and herbivore resistance. *Curr. Opin. Plant Biol.* 3: 309-314.

Felton, G.W., Korth, K.L., Bi, J.L., Wesley, S.V., Huhman, D.V., Mathews, M.C., Murphy, J.B., Lamb, C. and Dixon, R.A. (1999). Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. *Curr. Biol.* 9: 317-320.

Fritzsche-Hoballah, M.E. and Turlings, T.C.J. (2001). Experimental evidence that plants under caterpillar attack may benefit from attracting parasitoids. *Evol. Ecol. Res.* 3: 553-565.

Geervliet, J.B.F., Posthumus, M.A., Vet, L.E.M. and Dicke, M. (1997). Comparative analysis of headspace volatiles from different caterpillar-infested or uninfested food plants of *Pieris* species. *J. Chem. Ecol.* 23: 2935-2954.

Geervliet, J.B.F., Vet, L.E.M., Dicke, M., 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecul*a. *Entomol. Exp. Appl.* 73: 289-297.

Geervliet, J.B.F., Vet, L.E.M., Dicke, M., 1996. Innate responses of the parasitoids *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae) to volatiles from different plant-herbivore complexes. *J. Insect Beh.* 9: 525-538.

Geervliet, J.B.F., Vreugdenhil, A.I., Dicke, M. and Vet, L.E.M. (1998). Learning to discriminate between infochemicals from different plant-host complexes by the parasitoids *Cotesia glomerata* and *C. rubecula*. *Entomol. Exp. Appl.* 86: 241-252.

Gershenzon, J. and Croteau, R. (1991). Terpenoids. In: G.A. Rosenthal & M.R. Berenbaum: Herbivores: their interactions with secondary plant metabolites 1: 165-219.

Glazebrook, J. (2001). Genes controlling expression of defense responses in Arabidopsis - 2001 status. Curr. Opin. Plant Biol. 4: 301-308.

Gols, R., Posthumus, M.A. and Dicke, M. (1999). Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent Phytoseiulus persimilis. Entomol. Exp. Appl. 93: 77-86.

Groot, A.T., Dicke, M. (2001). Transgenic crops in an agro-ecological context: Multitrophic effects of insect-resistant plants. Wageningen University, Wageningen, The Netherlands.

Gundlach, H., Muller, M.J., Kutchan, T.M. and Zenk, M.H. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. USA 89: 2389-2393.

Halitschke, R., Schittko, U., Pohnert, G., Boland, W. and Baldwin, I.T. (2001). Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. Plant Physiol. 125: 711-717.

Harvey, J.A. (2000). Dynamic effects of parasitism by an endoparasitoid wasp on the development of two host species: implications for host quality and parasitoid fitness. Ecol. Entomol. 25: 267-278.

Horiuchi, J.-I., Arimura, G.-I., Ozawa, R., Shimoda, T., Takabayashi, J. and Nishioka, T. (2001). Exogenous ACC enhances volatile production mediated by jasmonic acid in lima bean leaves. FEBS lett. 509: 332-336.

Kahl, J., Siemens, D.H., Aerts, R.J., Gabler, R., Kuhnemann, F., Preston, C.A. and Baldwin, I.T. (2000). Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. Planta 210: 336-342.

Karban, R. and Baldwin, I.T. (1997). Induced Responses to Herbivory. Chicago, USA: The University of Chicago Press Ltd.

Kessler, A. and Baldwin, I.T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. Science 291: 2141-2144.

Kessler, A. and Baldwin, I.T. (2002). Plant responses to insect herbivory: the merging molecular analysis. Annu. Rev. Plant Biol. 53: 299-328.

Koch, T., Krumm, T., Jung, V., Engelberth, J. and Boland, W. (1999). Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. Plant Physiol. 121: 153-162.

Krischik, V. A., Barbosa, P., Reichelderfer, C. F. (1988). Three trophic level interactions: allelochemicals, Manduca sexta (L.), and Bacillus thuringiensis var. kurstaki Berliner. Environ. Entomol. 17: 476-482.

Laudert, D. and Weiler, E.W. (1998). Allene oxide synthase: a major control point in Arabidopsis thaliana octadecanoid signalling. Plant J. 15: 675-684.

Léon, J., Rojo, E., Sanchez-Serrano, J.J. 2001. Wound signalling in plants. J. Exp. Bot. 52:1-9.

Mattiacci, L., Dicke, M. and Posthumus, M.A. (1994). Induction of parasitoid attracting synomone in Brussels sprouts plants by feeding of Pieris brassicae larvae: role of mechanical damage and herbivore elicitor. J. Chem. Ecol. 20: 2229-2247.

Mattiacci, L., Dicke, M. and Posthumus, M.A. (1995). ß-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. Proc. Natl. Acad. Sci. USA. 92: 2036-2040.

Mauch-Mani, B. and Slusarenko, A.J. (1996). Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of Arabidopsis to Peronospora parasitica. Plant Cell 8: 203-212.

Mauricio, R. (1998). Costs of resistance to natural enemies in field populations of the annual plant Arabidopsis thaliana. Am. Naturalist 151: 20-28.

McCall, P.J., Turlings, T.C.J., Loughrin, J., Proveaux, A.T. and Tumlinson, J.H. (1994). Herbivore-induced volatile emissions from cotton (Gossypium hirsutum L.) seedlings. J. Chem. Ecol. 20: 3039-3050.

McCloud, E.S. and Baldwin, I.T. (1997). Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in Nicotiana sylvestris. Planta 203: 430-435.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E. and Browse, J. (1997). Jasmonate is essential for insect defense Arabidopsis. Proc. Natl. Acad. Sci. USA 94: 5473-5477.

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D. and Koornneef, M. (1998). Arabidopsis thaliana: A model plant for genome analysis. Science 282: 662&.

Métraux, J.P. (2001). Systemic acquired resistance and salicylic acid: current state of knowledge. Eur. J. Plant Pathol. 107: 13-18.

MitchellOlds, T., Gershenzon, J., Baldwin, I. and Boland, W. (1998). research focus - Chemical ecology in the molecular era. Trends Plant Sci. 3: 362-365.

Moran, P.J. and Thompson, G.A. (2001). Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways. Plant Physiol. 125: 1074-1085.

Musser, R.O., Hum-Musser, S.M., Eichenseer, H., Peiffer, M., Ervin, G., Murphy, J.B., Felton, G.W., 2002. Caterpillar saliva beats plant defences: A new weapon emerges in the evolutionary arms race between plants and herbivores. Nature 416: 599-600

O'Donnell, P., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O. and Bowles, D.J. (1996). Ethylene as a signal mediating the wound response of tomato plants. Science 274: 1914-1917.

Ozawa, R., Arimura, G., Takabayashi, J., Shimoda, T. and Nishioka, T. (2000). Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. Plant Cell Physiol. 41: 391-398.

Pare, P.W. and Tumlinson, J.H. (1997). De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol. 114: 1161-1167.

Pare, P.W. and Tumlinson, J.H. (1998). Cotton volatiles synthesized and released distal to the site of insect damage. Phytochemistry 47: 521-526.

Piel, J., Atzorn, R., Gabler, R., Kuhnemann, F. and Boland, W. (1997). Cellulysin from the plant parasitic fungus Trichoderma viride elicits volatile biosynthesis in higher plants via the octadecanoid signalling cascade. FEBS Lett. 416: 143-148.

Pieterse, C.M.J., Ton, J. and Van Loon, L.C. (2001a). Cross-talk between plant defence signalling pathways: boost or burden? AgBiotechNet 3: 1-8.

Pieterse, C.M.J., Van Pelt, J.A., Van Wees, S.C.M., Ton, J., Leon Kloosterziel, K.M., Keurentjes, J.J.B., Verhagen, B.W.M., Knoester, M., Van der Sluis, I., Bakker, P. et al. (2001b). Rhizobacteria-mediated induced systemic resistance: Triggering, signalling and expression. Eur. J. Plant Pathol. 107: 51-61.

Pieterse, C.M.J., vanWees, S.C.M., vanPelt, J.A., Knoester, M., Laan, R., Gerrits, N., Weisbeek, P.J. and vanLoon, L.C. (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell 10: 1571-1580.

Potting, R.P.J., Vet, L.E.M. and Dicke, M. (1995). Host Microhabitat Location by Stem-Borer Parasitoid Cotesia Flavipes - the Role of Herbivore Volatiles and Locally and Systemically Induced Plant Volatiles. J. Chem. Ecol. 21: 525-539.

Preston, C.A., Lewandowski, C., Enyedi, A.J. and Baldwin, I.T. (1999). Tobacco mosaic virus inoculation inhibits wound-induced jasmonic acid-mediated responses within but not between plants. Planta 209: 87-95.

Reymond, P., Weber, H., Damond, M. and Farmer, E.E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell 12: 707-719.

Rose, U.S.R., Lewis, W.J. and Tumlinson, J.H. (1998). Specificity of systemically released cotton volatiles as attractants for specialist and generalist parasitic wasps. J. Chem. Ecol. 24: 303-319.

Rose, U.S.R., Manukian, A., Heath, R.R. and Tumlinson, J.H. (1996). Volatile semiochemicals released from undamaged cotton leaves - A systemic response of living plants to caterpillar damage. Plant Physiol.111: 487-495.

Ryals, J., Lawton, K.A., Delaney, T.P., Friedrich, L., Kessmann, H., Neuenschwander, U., Uknes, S., Vernooij, B. and Weymann, K. (1995). Signal transduction in systemic acquired resistance. Proc. Natl. Acad. Sci. USA 92:4202-4205.

Ryan, C.A. (2000). The systemin signaling pathway: differential activation of plant defensive genes. Biochim. Biophys. Acta 1477: 112-121/

Sabelis, M.W., Van Baalen, M., Bakker, F.M., Bruin, J., Drukker, B., Egas, M., Janssen, A.R.M., Lesna, I.K., Pels, B., Van Rijn, P.C.J. et al. (1999). The evolution of direct and indirect plant defence against herbivorous arthropods. In *Herbivores Between Plants And Predators* (H. Olff, V.K. Brown, and R.H. Drent, eds.), Blackwell Science Ltd., Oxford, UK, pp. 109-166.

Schaller, F. (2001). Enzymes of the biosynthesis of octadecanoid-derived signalling molecules. *J. Exp. Bot.* 52: 11-23.

Schaller, F. and Weiler, E.W. (2002). Wound- and mechanical signalling. In *Plant Signal Transduction*, Scheel D. and Wasternack C. (eds). Oxford University Press, Oxford, UK, pp. 20-44.

Schoonhoven, L.M., Jermy, T. and Van Loon, J.J.A. (1998). *Insect-plant biology, from physiology to evolution*. Chapman & Hall, London, UK.

Shiojiri, K., Takabayashi, J., Yano, S., Takafuji, A., 2000. Herbivore-species-specific interactions between crucifer plants and parasitic wasps (Hymenoptera : Braconidae) that are mediated by infochemicals present in areas damaged by herbivores. *Appl. Entomol. Zool.* 35: 519-524.

Shimoda, T. and Dicke, M. (2000). Attraction of a predator to chemical information related to nonprey: when can it be adaptive? Behavioral Ecology 11: 606-613.

Staswick, P.E., Su, W.P., Howell, S.H., 1992. Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proc. Natl. Acad. Sci. USA* 89: 6837-6840.

Staswick, P.E., Yuen, G.Y. and Lehman, C.C. (1998). Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus Pythium irregulare. *Plant J.* 15: 747-754.

Stintzi, A., Weber, H., Reymond, P., Browse, J. and Farmer, E.E. (2001). Plant defense in the absence of jasmonic acid: The role of cyclopentenones. *Proc. Natl. Acad. Sci. USA* 98: 12837-12842.

Stotz, H.U., Pittendrigh, B.R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A. and Mitchell Olds, T. (2000). Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of *Arabidopsis* against Egyptian cotton worm but not diamondback moth. Plant-Physiology 124: 1007-1017.

Stotz, H.U., Koch, T., Biedermann, A., Weniger, K., Boland, W., Mitchell-Olds, T. (2002) Evidence for regulation of resistance in *Arabidopsis* to Egyptian cotton worm by salicylic and jasmonic acid signalling pathways. *Planta*, 214, 648-652.

Stout, M.J., Fidantsef, A.L., Duffey, S.S. and Bostock, R.M. (1999). Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiol. Mol. Plant Pathol.* 54: 115-130.

Takabayashi, J., Dicke, M. and Posthumus, M.A. (1991). Variation in composition of predator-attracting allelochemical emitted by herbivore-infested plants: relative influence of plant and herbivore. *Chemoecology* 1-6.

Takabayashi, J., Takahashi, S., Dicke, M. and Posthumus, M.A. (1995). Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. *J. Chem. Ecol.* 21: 273-287.

Tallamy, D.W., and Raupp M.J. (1991), *Phytochemical induction by herbivores.* John Wiley, New York, USA, pp. 105-132.

Thaler, J.S. (1999). Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399: 686-688.

Thomma, B., Eggermont, K., Penninckx, I., Mauch Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. USA* 95: 15107-15111.

Tjallingii, W.F., Hogen Esch, T., 1993. Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. Physiological Entomology, 18: 317-328.

Tomczyk, A., Kropczynska, D., 1985. Effects on the Host Plant. In *Spider mites, their biology, natural enemies and control* vol 1A (W. Helle, M. W. Sabelis, eds.), Elsevier, Amsterdam, The Netherlands, pp 317-329.

Turlings, T.C.J., Bernasconi, M., Bertossa, R., Bigler, F., Caloz, G. and Dorn, S. (1998). The induction of volatile emissions in maize by three herbivore species with different feeding habits: Possible consequences for their natural enemies. *Biol. Control* 11: 122-129.

Turlings, T.C.J. and Tumlinson, J.H. (1992). Systemic release of chemical signals by herbivore-injured corn. *Proc. Natl. Acad. Sci. USA* 89: 8399-8402.

Turlings, T.C.J., Tumlinson, J.H. and Lewis, W.J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 30: 1251-1253.

Vancanneyt, G., Sanz, C., Farmaki, T., Paneque, M., Ortego, F., Castanera, P. and Sanchez Serrano, J.J. (2001). Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. *Proc. Natl. Acad. Sci. USA* 98: 8139-8144.

Van Loon, J.J.A., de Boer, J.G. and Dicke, M. (2000). Parasitoid-plant mutualism: parasitoid attack of herbivore increases plant reproduction. *Entomol. Exp. Appl.* 97: 219-227.

Van Wees, S.C.M., De Swart, E.A.M., Van Pelt, J.A., Van Loon, L.C., Pieterse, C.M.J. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 97: 8711-8716,

Vet, L.E.M. and Dicke, M. (1992). Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37: 141-172.

Walling, L.L. (2000). The myriad plant responses to herbivores. *J. Plant Growth Regulation* 19: 195-216.

Zhang, Y.L., Fan, W.H., Kinkema, M., Li, X. and Dong, X.N. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proc. *Natl. Acad. Sci. USA* 96: 6523-6528.

Nederlandse inleiding en samenvatting

Remco M.P. van Poecke

"Wat ik zeggen wil is kort, maar ik meen het."

André Hazes - Ik meen het

Nederlandse inleiding en samenvatting

INDIRECTE VERDEDIGING VAN DE PLANT *ARABIDOPSIS* TEGEN HERBIVORE INSECTEN:

HET COMBINEREN VAN GEDRAGSSTUDIES VAN SLUIPWESPEN EN CHEMISCHE ANALYSES VAN VLUCHTIGE STOFFEN MET EEN MOLECULAIR GENETISCHE BENADERING

Deze 'Nederlandse inleiding en samenvatting' is geschreven voor niet-vakgenoten. Het is een vertaling van delen van het eerste en het laatste hoofdstuk van dit proefschrift, waarbij vaktaal zoveel mogelijk is vervangen door algemeen taalgebruik.

1. Het gevecht om het bestaan

Planten hebben geen gemakkelijk leven. Zodra ze uit zaad ontkiemen moeten ze kou, wind, te veel of te weinig water, het branden van de zon of het striemen van de regen weerstaan. En als ze niet vertrapt worden onder de hoeven van grazers of geplaagd worden door ziektes, dan zijn het wel insecten die hun bladeren, stengels, wortels of bloemen aanvreten. Terwijl ze zich tegen deze aanvallen verdedigen, moeten ze zoeken naar water, nutriënten en vaak ook bestuivers om nieuw zaad te maken. Aangezien planten niet weg kunnen rennen, moeten planten zich aanpassen en verdedigen om te overleven.

Daarom hebben planten een heel scala aan verdedigingsmechanismen. Sommige van deze verdedigingsmechanismen zijn permanent aanwezig, ook al wordt de plant niet aangevallen. Deze *constitutieve* verdediging is de eerste verdedigingslinie van een plant tegen aanvallen van herbivoren of andere ongunstige omstandigheden. Voor het maken van deze verdedigingsmechanismen heeft de plant water, nutriënten, licht en koolzuurgas uit zijn omgeving nodig. Dit geeft ook meteen een nadeel van constitutieve verdediging aan: er zijn kosten aan verbonden. De grondstoffen en energie geïnvesteerd in verdediging kunnen meestal niet gebruikt worden voor groei of voortplanting.

2. Induceerbare verdediging

De kosten van constitutieve verdediging vormen waarschijnlijk één van de redenen waarom een plant, naast constitutieve verdediging, vaak ook induceerbare verdedigingsmechanismen gebruikt. Deze verdedigingsmechanismen worden alleen aangeschakeld wanneer een plant ze nodig heeft. Een voorbeeld is de ophoping van suikers als verdediging tegen bevriezing en de productie van toxische stoffen (toxines) na vraat door herbivoren. Alhoewel de kostenreductie waarschijnlijk een stimulans was voor de evolutie van induceerbare verdediging, zijn er nog andere voordelen. Zo kunnen herbivoren die continu worden blootgesteld aan toxines zich vaak sneller aanpassen aan deze stoffen dan herbivoren die blootgesteld worden aan wisselende concentraties toxines.

Als induceerbare verdediging zoveel voordelen heeft ten opzichte van constitutieve verdediging, waarom zijn dan niet alle verdedigingsmechanismen induceerbaar? Blijkbaar zijn er ook nadelen verbonden aan induceerbare verdediging. Eén nadeel is dat het tijd kost om induceerbare verdediging in stelling te brengen. In die tijd kan de plant al aanzienlijke schade hebben opgelopen.

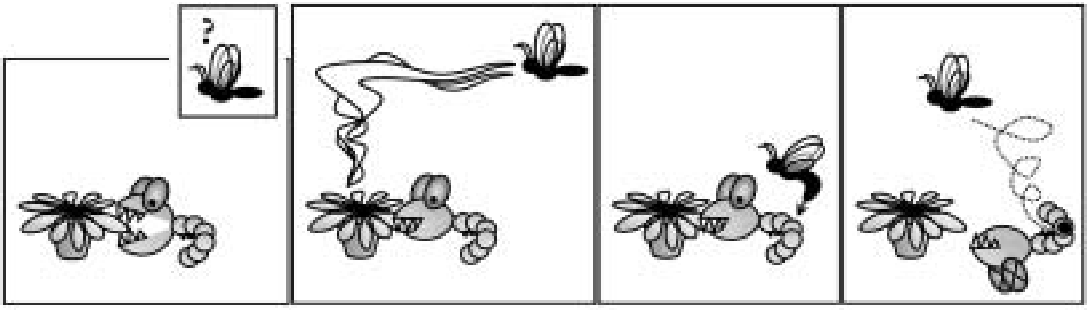
3. Signaaltransductie

In tegenstelling tot constitutieve verdediging heeft het gebruik van induceerbare verdediging meer interactie van de plant met z'n omgeving nodig dan alleen het verkrijgen van grondstoffen en energie. De plant moet veranderingen in z'n omgeving (signalen) kunnen waarnemen, of dit nu een daling van de omgevingstemperatuur, een aanval van herbivore insecten, of andere veranderingen zijn. Nadat deze veranderingen zijn waargenomen, moet deze informatie worden verwerkt en uiteindelijk omgezet worden in een reactie die de plant (enige) bescherming biedt. Het verwerken van deze signalen wordt signaaltransductie genoemd, de routes in de plant via welke dit gebeurt signaaltransductie-routes.

Veel signaaltransductie-routes in de plant zijn afhankelijk van plantenhormonen. Verscheidene van deze plantenhormonen zijn inmiddels ontdekt en hun rol in de verdediging van een plant vastgesteld. Voorbeelden van dergelijke hormonen zijn jasmonzuur (JA) en salicylzuur (SA). Er zijn echter meer verschillende, induceerbare verdedigingsmechanismen dan dat er verschillende plantenhormonen zijn. Dit betekent dat niet bij elk verdedigingsmechanisme een specifiek plantenhormoon hoort. Bovendien vervullen veel plantenhormonen, naast hun rol in verdediging, ook rollen in andere processen, zoals groei en bloemontwikkeling. Om zo'n verscheidenheid aan functies met een beperkt aantal plantenhormonen te bewerkstelligen zijn er verschillende mogelijkheden. Eén van deze mogelijkheden is interactie tussen hormonen. Als een bepaalde reactie de gecombineerde activiteit van verschillende hormonen nodig heeft, zijn er veel meer verschillende reacties mogelijk met een beperkt aantal hormonen. Dit houdt echter ook in dat verschillende reacties afhankelijk zijn van hetzelfde hormoon en daardoor elkaar positief of negatief kunnen beïnvloeden. Alhoewel verscheidene plantenhormonen zijn ontdekt en hun interacties soms gedeeltelijk worden begrepen, is de manier waarop signaaltransductie-routes elkaar beïnvloeden en hoe dit resulteert in de verscheidenheid aan reacties grotendeels onbekend.

3- *Indirecte verdediging*

De verdediging van planten tegen herbivore insecten omvat vele verschillende mechanismen. Sommige van deze mechanismen hebben een directe uitwerking op de herbivoor, zoals bijvoorbeeld de productie van toxische of afstotende stoffen. Andere mechanismen werken indirect tegen herbivoren, door gebruik te maken van natuurlijke vijanden van herbivoren, namelijk carnivoren. Carnivoren kunnen zowel predatoren als parasitoïden zijn. Predatoren eten de herbivoren of hun eieren, parasitoïden leggen hun eieren in of op herbivoren of hun eieren. Zodra de eieren van de parasitoïden uitkomen beginnen de larven de herbivoor (of de eieren van de herbivoor) op te eten. De plant kan de effectiviteit van de carnivoren vergroten, bijvoorbeeld door ze extra voedsel of schuilplaatsen te bieden, of door de carnivoren te informeren over de aanwezigheid van herbivoren. Herbivoren staan vaak onder evolutionaire druk om niet door carnivoren te worden ontdekt. Planten kunnen de carnivoren helpen de herbivoren te vinden, bijvoorbeeld door het produceren en uitscheiden van vluchtige stoffen zodra een herbivoor van ze begint te eten (zie figuur 1). Deze informatie kan zo gedetailleerd zijn, dat het zelfs een indicatie geeft van de soort herbivoor die de plant aanvalt. Dit is belangrijk omdat veel carnivoren specialisten zijn, die slechts één of enkele soorten herbivoren eten.



Een plant wordt aangevallen door een rups. Ondertussen is een sluipwesp op zoek naar zijn gastheer (de rups).

Zodra de rups de plant beschadigt, begint de plant vluchtige stoffen uit te scheiden. De sluipwesp gebruikt dit 'geursignaal' om de rups te vinden.

Nu de sluipwesp de rups heeft gevonden legt ze er een eitje in.

Na verloop van tijd kruipt de larf van de sluipwesp uit de rups en verpopt zich tot een nieuwe sluipwesp. De rups sterft en kan de plant dus niet verder beschadigen.

Figuur 1. *Informatie-verstrekking door de plant, in de vorm van vluchtige stoffen, vergroot de effectiviteit van carnivoren (in dit geval een sluipwesp).*

Omdat de productie van deze vluchtige stoffen geïnduceerd wordt door herbivorenvraat, is het herkennen van vraatschade en het verwerken van deze informatie door de plant noodzakelijk. Herkenning van vraatschade door de plant omvat reacties op verschillende signalen. Eén ervan is verwonding. Zodra een herbivoor van een plant eet wordt er plantenweefsel beschadigd. Deze beschadiging resulteert in de productie van vluchtige stoffen. Andere herbivoor-afhankelijke signalen zijn stoffen die voorkomen in spuug van herbivoren.

Verschillende stoffen, geïsoleerd uit spuug van rupsensoorten, kunnen de productie van vluchtige stoffen door de plant induceren.

Kennis over hoe de bovenstaande signalen door een plant worden herkend en hoe deze informatie wordt verwerkt is beperkt. Het plantenhormoon jasmonzuur (bekend van zijn rol in signaaltransductie van verwonding en in de inductie van directe verdediging tegen herbivore insecten) speelt hierbij een rol. Recentelijk is ook een rol voor salicylzuur (betrokken bij de geïnduceerde verdediging van planten tegen vele soorten ziektes) in de herbivoor-geïnduceerde emissie van vluchtige stoffen gesuggereerd. Hoe deze plantenhormonen de productie van deze vluchtige stoffen beïnvloeden is nog onbekend.

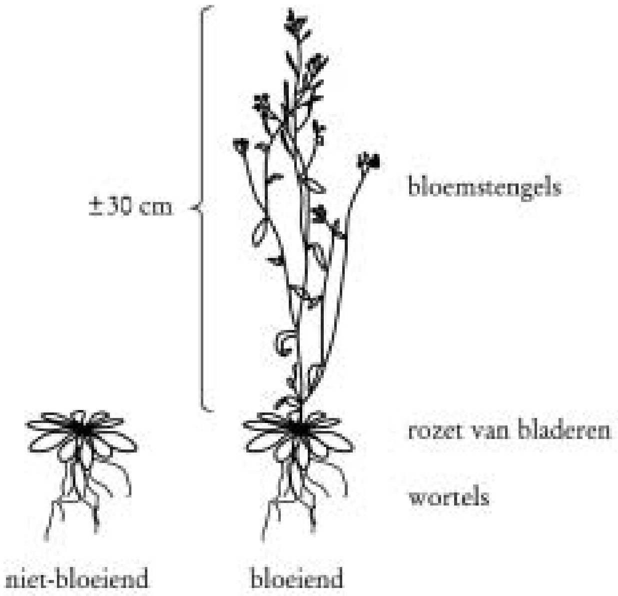
5. *Arabidopsis thaliana* als model-plant

Om meer te weten te komen van signaaltransductie in herbivoor-geïnduceerde productie van vluchtige stoffen heb ik *Arabidopsis thaliana* (zandraket) geïntroduceerd in dit onderzoeksveld. *Arabidopsis* behoort tot de Brassicaceae plantenfamilie (kruisbloemigen), waartoe ook planten zoals kool en mosterd behoren. Deze plant wordt veel gebruikt in onderzoek naar signaaltransductie-routes, waaronder signaaltransductie-routes betrokken bij geïnduceerde verdedigingsmechanismen. Waarom deze plant wordt gebruikt heeft verscheidene redenen. Allereerst is *Arabidopsis* makkelijk te kweken. De complete levenscyclus van kieming van het zaad tot de vorming van bloemen en de productie van nieuw zaad kan in zes weken worden voltooid. De plant is klein en simpel van structuur (zie figuur 2), heeft geen ingewikkelde groeivereisten, produceert duizenden zaden per plant en is makkelijk te kruisen. Bovendien heeft het een klein genoom[1] wat genetische studies vergemakkelijkt. De hele genoomsequentie[2] van *Arabidopsis* is bekend. Het genoom van *Arabidopsis* is gemakkelijk te veranderen via mutaties en transformaties. Verder komt *Arabidopsis* wijd verspreid voor in Eurasië, Noord-Afrika en Noord-Amerika. Hierdoor zijn er veel verschillende ecotypes (*Arabidopsis* planten die op verschillende plaatsen zijn gevonden en genetisch

---

[1] Het genoom omvat alle genetische informatie die zich in de kern van een plantencel bevindt. Het bestaat uit chromosomen (gemaakt van DNA) waarop genen liggen. Het genoom kan worden gezien als een taal, waarbij de genen de woorden zijn. Het samenspel tussen deze woorden vormt, zodra ze gelezen worden, een verhaal. Afhankelijk van de combinatie van woorden kun je verschillende verhalen maken. Op dezelfde wijze zorgt een verschillend samenspel van genen voor verschillende processen in de plant. Zoals het makkelijker is om een taal met weinig woorden te leren, zo is het ook makkelijker om een kleiner genoom te begrijpen.

[2] Woorden van een taal bestaan uit verschillende letters en de verschillende letters vormen het alfabet. Het 'alfabet' van het genoom is beperkt, het kent maar vier letters (A, T, G, en C). Met deze vier letters kan echter een uitgebreide woordenschat worden gemaakt (denk bijvoorbeeld aan computertaal die slechts uit enen en nullen bestaat). Zoals de volgorde van verschillende letters een woord vormt, zo vormt de volgorde van letters van het 'genoomalfabet' een gen. De volgorde van deze letters heet de sequentie van gen. Op die manier bestaat het hele genoom uit enorme reeksen van A's, T's, G's en C's. Dit is de genoomsequentie.



Figuur 2 Tekening van de plant *Arabidopsis thaliana* (zandraket). De experimenten met *Arabidopsis* die worden beschreven in dit proefschrift zijn altijd gedaan met niet-bloeiende planten

verschillend zijn). Een ander voordeel is dat *Arabidopsis* door veel verschillende soorten herbivore insecten en pathogenen (ziekteverwekkers) wordt belaagd.

Deze vele voordelen van *Arabidopsis* hebben er toe geleid dat er een enorme variëteit aan wild-type, transgene en mutant-planten is. Sommige hiervan hebben afwijkende of geblokkeerde signaaltransductie-routes. Dergelijke planten zijn erg waardevol bij het bestuderen van deze signaaltransductie-routes. Signaaltransductie in de verdediging van *Arabidopsis* tegen pathogenen is dan ook al uitvoerig bestudeerd en recentelijk is *Arabidopsis* ook gebruikt in studies naar signaaltransductie in de directe verdediging van planten tegen herbivore insecten. Aanvullend bij de bovengenoemde voordelen is er daarom veel informatie over signaaltransductie in geïnduceerde reacties van *Arabidopsis.* Deze informatie kan gebruikt worden om mijn resultaten met betrekking tot signaaltransductie in indirecte verdediging tegen insecten te vergelijken met andere signaaltransductie-routes, zoals die betrokken bij verwonding of verdediging tegen pathogenen.

6. Plant-herbivoor-parasitoid interacties: *Arabidopsis-Pieris-Cotesia*

Kruisbloemigen worden door verschillende soorten herbivore insecten aangevallen. Er is al veel onderzoek gedaan naar de interactie tussen kruisbloemigen en vlinders (en rupsen) van het geslacht *Pieris*. Het is bekend dat *Pieris* rupsen de productie van vluchtige stoffen door kruisbloemigen kan induceren. Zo produceren spruitkoolplanten (*Brassica oleracea*) na *Pieris*-vraat vluchtige stoffen die *Cotesia* sluipwespen aantrekken. In mijn onderzoek heb ik voornamelijk gewerkt met rupsen van het kleine koolwitje (*Pieris rapae*) en hun natuurlijke vijanden, *Cotesia rubecula* sluipwespen.

Rupsen van het kleine koolwitje eten alleen van planten die glucosinolaten bevatten en dit zijn voornamelijk kruisbloemigen. Het kleine koolwitje komt voor in Eurasië, Noord-Afrika, Noord-Amerika en Australië, zodat er een grote overlap is in de geografische verdeling tussen *Arabidopsis* en het kleine koolwitje. Inderdaad worden rupsen van het kleine koolwitje op *Arabidopsis* gevonden. Het kleine koolwitje is een solitaire vlindersoort, wat betekent dat de vlinder in plaats van een eipakket slechts één ei per keer legt. Dit houdt dan vaak ook in dat er slechts één ei per plant wordt gelegd.

De sluipwesp *Cotesia rubecula* is gespecialiseerd op rupsen van het kleine koolwitje. Deze sluipwesp gebruikt voornamelijk de vluchtige stoffen van planten om haar gastheer (rupsen van het kleine koolwitje dus) van een afstand te vinden. Het kleine koolwitje heeft vijf larvale stadia, waarvan het eerste, tweede en in mindere mate derde stadium door *C. rubecula* geparasiteerd kunnen worden. *Cotesia rubecula* is ook een solitaire soort, wat inhoudt dat de sluipwesp slechts één ei per keer legt. Per rups kan zich ook slechts één *C. rubecula* ei tot sluipwesp ontwikkelen. De larven van de sluipwesp komen uit de rups gekropen op het moment dat deze zich in het vierde larvale stadium bevindt. Vervolgens verpoppen de sluipwesp-larven zich buiten hun gastheer. De rups sterft snel nadat de sluipwesp-larve eruit is gekropen. Dit is van belang voor de plant, aangezien ongeparasiteerde rupsen de grootste schade toedienen als ze in hun vijfde larvale stadium zijn. Dit betekent dat een plant minder wordt beschadigd en meer zaad kan produceren wanneer hij wordt aangevreten door een geparasiteerde rups in vergelijking met een ongeparasiteerde rups. Dat dit zo is werd voor het eerst aangetoond voor *Arabidopsis.*

7. Vraagstellingen behandeld in dit proefschrift

Kort samengevat weten we dat planten zich op indirecte wijze kunnen verdedigen tegen herbivore insecten door carnivoren zoals sluipwespen aan te trekken. Aangezien dit een geïnduceerd verdedigingsmechanisme is, moet er ook sprake zijn van signaaltransductie in de plant. De kennis van de signaaltransductie die betrokken is bij de door herbivoren geïnduceerde productie van vluchtige stoffen door de plant is beperkt. We weten wel dat de plantenhormonen jasmonzuur en salicylzuur waarschijnlijk een rol spelen. Deze plantenhormonen zijn ook betrokken bij geïnduceerde directe verdediging van de plant tegen herbivore insecten en pathogenen. Het gebruik van *Arabidopsis thaliana*, een belangrijke modelplant in de hedendaagse plantenwetenschappen, is ideaal om via een moleculair-genetische methode[3] meer te weten te komen over de signaaltransductie in indirecte verdediging tegen herbivore insecten en hoe deze signaaltransductie in relatie staat tot signaaltransductie in directe verdediging. Het

---

[3] In mijn onderzoek houdt dit in dat ik kijk naar genexpressie (dit wordt later uitgelegd) en gebruik maak van genetisch gemodificeerde planten, namelijk mutanten en transgenen.

belang van zo'n moleculair-genetische methode is al door verschillende wetenschappers benadrukt. Dit proefschrift is één van de eerste publicaties die de resultaten behaald met een dergelijke methode beschrijft voor indirecte verdediging. In dit proefschrift behandel ik de volgende vragen:

1. Is *Arabidopsis* een geschikte modelplant om indirecte verdediging, in het bijzonder de inductie van sluipwesp-aantrekkende geurstoffen, te bestuderen?
2. Zijn de plantenhormonen jasmonzuur en salicylzuur inderdaad betrokken bij de signaaltransductie in indirecte verdediging van *Arabidopsis* en zo ja, hoe?
3. Wat gebeurt er stroomafwaarts, na de rol van JA en SA, in deze signaaltransductie en wat is de relatie met andere geïnduceerde verdedigingsmechanismen, zoals die tegen pathogenen?

8. Inhoud van dit proefschrift

Voordat ik deze vragen in mijn proefschrift behandel wordt in hoofdstuk 2 eerst een overzicht gegeven van wat er over signaaltransductie in directe en indirecte verdediging van planten tegen herbivoren bekend is.

8.1 Vraag 1: Is *Arabidopsis* een geschikte modelplant om indirecte verdediging te bestuderen?

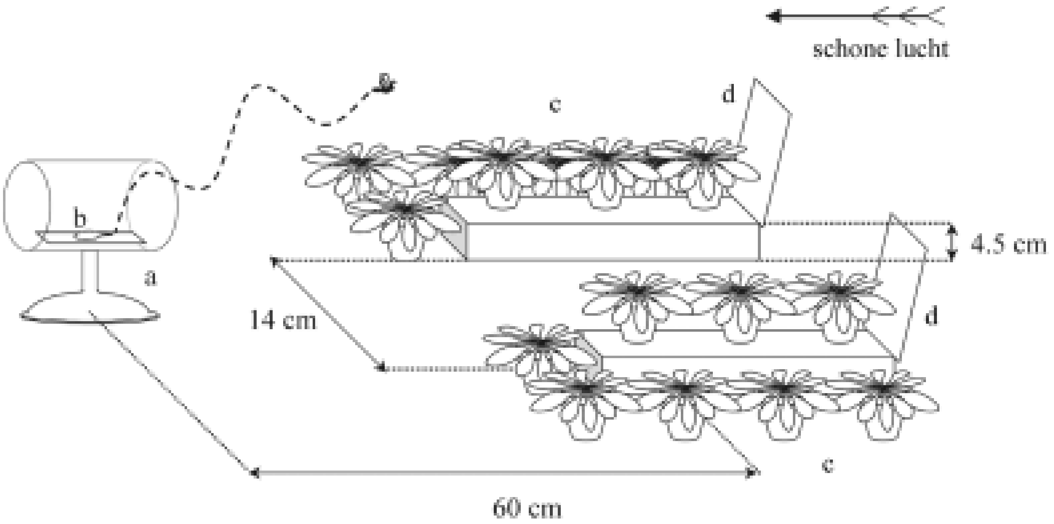
Om deze vraag te kunnen beantwoorden moeten we eerst weten aan welke eisen een modelplant moet voldoen:

1. De modelplant moet de eigenschap bezitten die men wil bestuderen.
2. Deze eigenschap moet vergelijkbaar zijn tussen de modelplant en de plantensoorten waarvoor de plant model staat.
3. Het moet makkelijker zijn de eigenschap in de modelplant te bestuderen dan in andere plantensoorten.
4. Andere, verwante eigenschappen worden ook bestudeerd in de modelplant.

De laatste twee eisen zijn al behandeld in paragraaf 5, dus resten de eerste twee eisen.

8.1.1 De modelplant moet de eigenschap bezitten die men wil bestuderen

Om indirecte verdediging in *Arabidopsis* te bestuderen moet eerst aangetoond worden dat *Arabidopsis* meer aantrekkelijk wordt voor carnivoren wanneer de plant door herbivoren wordt belaagd. In hoofdstuk 3 heb ik aangetoond dat *Cotesia rubecula* vrouwtjes een sterke voorkeur hebben voor geuren van *Arabidopsis* planten die worden aangevreten door rupsen van het kleine koolwitje ten opzichte van geuren van onbeschadigde *Arabidopsis* planten. Dergelijke keuze-experimenten zijn uitgevoerd in een windtunnel, waarbij ik meerdere planten per geurbron gebruikte (figuur 3). In hoofdstuk 3 laat ik ook zien dat een enkele *Arabidopsis* plant met een enkele, net uit het ei gekomen rups na verloop van tijd (3 dagen) aantrekkelijk wordt. Chemische analyse van de vluchtige stoffen toonde aan dat door rupsen aangevreten *Arabidopsis* planten stoffen uitscheiden die niet of in mindere mate worden uitgescheiden door onbeschadigde planten. Deze stoffen



Figuur 3 Set-up van een windtunnel experiment. In de windtunnel worden twee geurbronnen 60 cm windopwaarts van de loslaatcilinder (a) geplaatst. Een sluipwesp werd losgelaten op een rupsbeschadigd *Arabidopsis* blaadje zonder rupsen (b). Een geurbron (c) bestond uit acht *Arabidopsis* planten, waarvan er drie op een verhoogje van 4,5 cm stonden. Op dit verhoogje stond, windopwaarts van de planten, een rechthoek van plexiglas (d) om wat turbulentie te veroorzaken.

behoren tot verschillende groepen die door verschillende productieroutes in de plant worden gemaakt, zoals fenolen (o.a. methyl-salicylaat), terpeenachtigen (bijvoorbeeld de stof (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraeen, oftewel TMTT), nitrillen en zogenaamde 'groene geuren' die van vetzuren afkomstig zijn.

8.1.2 De te bestuderen eigenschap moet vergelijkbaar zijn tussen de modelplant en de plantensoorten waarvoor de plant model staat

Zoals al eerder werd genoemd, zijn verschillende signalen betrokken bij de herkenning van vraatschade door een plant. Allereerst kan de verwonding worden waargenomen en ten tweede zijn factoren uit het spuug van herbivoren betrokken bij deze herkenning. Dit laatste betekent ook dat een plant verschillend kan reageren op alleen verwonding (bijvoorbeeld met een schaar, zgn. mechanische verwonding) of verwonding gecombineerd met de factoren uit het spuug (wat dus bijvoorbeeld voorkomt tijdens rupsenvraat). Inderdaad is na bestudering van vele verschillende plantensoorten gebleken dat mechanische verwonding resulteert in de emissie van een ander mengsel van vluchtige stoffen door de plant dan vraat door herbivoren. De verschillende plantensoorten kunnen ruwweg in twee groepen worden ingedeeld. De ene groep planten scheidt na herbivorenvraat stoffen uit die niet uitgescheiden worden door mechanisch beschadigde planten (er is sprake van *kwalitatief* verschillende mengsels van vluchtige stoffen). De andere groep planten scheidt voornamelijk stoffen uit na herbivorenvraat die ook door mechanisch beschadigde planten worden uitgescheiden, maar in andere hoeveelheden en andere onderlinge verhoudingen van de verschillende stoffen (er is sprake van *kwantitatief* verschillende mengsels van vluchtige stoffen). De resultaten uit hoofdstuk 3 tonen aan dat *Arabidopsis*, net als andere kruisbloemigen, behoort tot de tweede groep.

Hoewel de verschillen in vluchtige stoffen van mechanisch beschadigde en rupsaangevreten *Arabidopsis* planten niet erg groot lijken, zijn ze groot genoeg voor de sluipwespen om onderscheid te maken. In de windtunnel verkozen *C. rubecula* vrouwtjes de geuren van rupsbeschadigde boven die van mechanisch beschadigde *Arabidopsis* planten. Zodra ik mechanisch beschadigde planten behandelde met rupsenspuug, werden deze planten aantrekkelijker dan mechanisch beschadigde planten die niet met rupsenspuug waren behandeld. Sterker nog, de sluipwespen maakten geen onderscheid tussen mechanisch beschadigde planten met rupsenspuug en planten die door rupsen waren aangevreten (hoofdstuk 6). Dit is vergelijkbaar met resultaten behaald met andere plantensoorten.

Plantensoorten die *kwantitatief* verschillende mengsels van vluchtige stoffen uitscheiden na mechanische beschadiging of rupsen vraat, scheiden na vraat door verschillende soorten herbivoren meestal ook mengsels van vluchtige stoffen uit die *kwantitatief* verschillen. Van verschillende kruisbloemigen is aangetoond dat deze mengsels erg op elkaar lijken zodra bijvoorbeeld beschadiging door twee verschillende rupsensoorten wordt vergeleken. *Cotesia rubecula* wespen kunnen van afstand dan ook geen onderscheid maken tussen koolplanten aangevreten door rupsen van het kleine koolwitje en rupsen van een andere soort (*Plutella xylostella*) die voor *C. rubecula* niet geschikt zijn als gastheer. Sommige andere soorten sluipwespen kunnen dit echter wel. In hoofdstuk 7 van dit proefschrift tonen we aan dat *C. rubecula* vrouwtjes in de windtunnel geen onderscheid maken tussen *Arabidopsis* aangevreten door rupsen van het kleine koolwitje en rupsen van andere soorten (*P. xylostella* en *Spodoptera exigua*). Wel maken deze sluipwespen onderscheid tussen *Arabidopsis* aangevreten door rupsen van het kleine koolwitje en *Arabidopsis* aangevreten door herbivoren die niet tot de vlinders behoren en op een andere manier de plant beschadigen, zoals bladluizen (*Myzus persicae*), die de sapstroom van de plant aftappen en spintmijten (*Tetranychus urticae*), die individuele bladcellen leegzuigen.

Kort samengevat: dit proefschrift bevat vele gegevens die aantonen dat - met betrekking tot rupsenvraat-geïnduceerde vluchtige stoffen en de aantrekking van *C. rubecula* - *Arabidopsis* vergelijkbaar reageert als andere planten en met name kruisbloemigen. Ik concludeer dan ook dat *Arabidopsis* een goede modelplant is om signaal-transductie in indirecte verdediging te bestuderen.

8.2 Zijn de plantenhormonen jasmonzuur en salicylzuur inderdaad betrokken bij de signaaltransductie in indirecte verdediging van *Arabidopsis* en zo ja, hoe?

8.2.1 Jasmonzuur

Wat betreft de signaaltransductie-routes betrokken bij rupsenvraat-geïnduceerde productie van vluchtige stoffen zijn er sterke aanwijzing dat jasmonzuur hierbij is betrokken. Zo gaat het niveau van JA in de plant omhoog na rupsenvraat (wat o.a. is aangetoond voor *Arabidopsis*) en resulteert bespuiting van planten met JA in de productie van vluchtige stoffen en in de aantrekking van carnivoren. In hoofdstuk 4 en 5 laat ik zien dat *Arabidopsis* planten bespoten met JA meer vluchtige stoffen uitscheiden en meer sluipwespen aantrekken dan onbespoten planten. De sluipwespen verkiezen echter rups-aangevreten planten boven JA-bespoten planten.

Het voordeel van het gebruik van *Arabidopsis* is dat je ook op een andere manier naar de rol van JA kunt kijken. Bijvoorbeeld door gebruik te maken van transgene planten waarin het niveau van JA niet omhoog gaat na rupsenvraat (deze planten worden S-12 planten genoemd). Wanneer rupsen van deze transgene planten eten zijn deze planten minder aantrekkelijk voor *C. rubecula* dan aangevreten wild-type planten (hoofdstuk 4). Chemische analyse van het mengsel vluchtige stoffen uitgescheiden door aangevreten S-12 planten liet zien dat deze planten inderdaad minder stoffen uitscheiden dan wild-type planten (hoofdstuk 5). Sommige stoffen in het mengsel van wild-type planten worden niet geïnduceerd door rupsenvraat, maar komen in gelijke mate voor in het mengsel van onbeschadigde wild-type planten. Vreemd genoeg werden ook deze stoffen in mindere mate uitgescheiden door S-12 planten. Dit betekent dat S-12 planten niet zozeer veranderd zijn in de door rupsen geïnduceerde productie van vluchtige stoffen als wel in de emissie van alle vluchtige stoffen. S-12 planten hebben geen bladharen, waardoor het bladoppervlak kleiner is. Bovendien lijkt het erop dat deze planten ook minder huidmondjes hebben, die betrokken zijn bij de uitwisseling van vluchtige stoffen tussen plant en omgeving. Kortom, dat S-12 planten minder aantrekkelijk zijn voor *C. rubecula* komt waarschijnlijk doordat de veranderde jasmonzuur niveaus in deze planten invloed hebben gehad op de vorming van huidmondjes en bladharen en niet omdat deze planten veranderd zijn in de signaaltransductie die leidt tot rupsenvraat-geïnduceerde productie van vluchtige stoffen.

8.2.2 Salicylzuur

De aanwijzingen dat salicylzuur betrokken is bij rupsenvraat-geïnduceerde productie van vluchtige stoffen zijn beperkter. Allereerst wordt in veel gevallen methyl-salicylaat (MeSA), een meer vluchtige vorm van salicylzuur, vaak gevonden in het mengsel van vluchtige stoffen uitgescheiden door aangevreten planten. Verder is het zo dat behandeling van Lima boon, een andere modelplant, met MeSA resulteert in de emissie van bepaalde stoffen die ook door herbivorenvraat wordt geïnduceerd (met name emissie van de stof TMTT).

Arabidopsis planten bespoten met SA waren niet aantrekkelijker voor *C. rubecula* dan onbespoten controle planten (hoofdstuk 4). Echter het gebruik van transgene planten die geen SA kunnen maken (*NahG* planten) toonde aan dat SA wel betrokken is bij rupsenvraat-geïnduceerde productie van vluchtige stoffen door *Arabidopsis*. Wanneer rupsen van *NahG* planten vreten zijn deze planten minder aantrekkelijk voor *C. rubecula* vergeleken met aangevreten wild-type planten (hoofdstuk 4). Chemische analyse van het mengsel vluchtige stoffen uitgescheiden door aangevreten *NahG* planten liet zien dat deze planten bepaalde stoffen minder uitscheiden dan wild-type planten, met name de stoffen MeSA en TMTT (hoofdstuk 5).

8.2.3 Genexpressie

Behalve naar gedrag van *C. rubecula* en de chemische analyse van de vluchtige stoffen heb ik ook naar genexpressie (het aflezen van genen door de plant) gekeken. Hiervoor heb ik een beperkt aantal genen geselecteerd die mogelijk betrokken zijn bij de geïnduceerde productie van vluchtige stoffen door *Arabidopsis*. Van zes van de acht genen die ik had geselecteerd werd de expressie geïnduceerd door rupsenvraat[4] (hoofdstuk 3). De expressie van vijf van deze zes genen heb ik ook bestudeerd in al dan niet aangevreten S-12 en *NahG* planten. Hoewel deze genen maar een zeer beperkt deel vormen van de genen betrokken bij geïnduceerde productie van vluchtige stoffen was het toch verrassend dat de expressie van deze vijf genen met een enkele uitzondering nauwelijks verschilde tussen wild-type, S-12 en *NahG* planten (hoofdstuk 5).

Gebaseerd op de bovenstaande resultaten heb ik in hoofdstuk 5 een model gepresenteerd waarin ik de rol van JA en SA in rupsenvraat-geïnduceerde productie van vluchtige stoffen door *Arabidopsis* beschrijf.

8.3 Wat gebeurt er stroomafwaarts, na de rol van JA en SA, in deze signaaltransductie en wat is de relatie met andere geïnduceerde verdedigingsmechanismen, zoals die tegen pathogenen?

Eén van de belangrijkste redenen voor het gebruik van *Arabidopsis* is dat er van *Arabidopsis* veel signaaltransductie mutanten of transgenen zijn. In dit opzicht is *Arabidopsis* uniek. Dit houdt in dat we behalve plantenhormonen ook allerlei andere factoren kunnen bestuderen die betrokken zijn bij signaaltransductie. Nadat bijvoorbeeld de niveaus van JA in de plant door rupsenvraat zijn verhoogd, moet er nog heel wat gebeuren voordat dit uiteindelijk leidt tot verhoogde productie van vluchtige stoffen. De verhoging van JA is dus slechts een signaal dat weer andere signalen genereert, die op hun beurt weer andere signalen genereren, enzovoort. Dit wordt ook wel een signaaltransductie-cascade genoemd. *Arabidopsis* is dus een uitstekende plant voor de studie van dergelijke signaaltransductie-cascades. Zo is er onder andere bekend dat in de geïnduceerde verdediging van *Arabidopsis* tegen pathogenen, de factoren NPR1 en JAR1 betrokken zijn bij de signalering stroomafwaarts van salicylzuur en/of jasmonzuur. Mutaties in deze factoren hebben dan ook hun invloed op deze verdediging. De mutant-planten *npr1-1* en *jar1-1* zijn gemuteerd in respectievelijk het *NPR1* en *JAR1* gen. In hoofdstuk 6 wordt aangetoond dat deze mutanten net zo aantrekkelijk zijn voor *C. rubecula* wanneer ze aangevreten zijn door rupsen van het kleine koolwitje vergeleken met wild-type planten. Kennelijk zijn de factoren NPR1 en JAR1 niet belangrijk voor de geïnduceerde indirecte verdediging van *Arabidopsis* tegen

---

[4] Als de expressie van genen wordt geïnduceerd, betekent dit dat ze vaker worden afgelezen. Na het aflezen van genen door de plant wordt deze informatie (in de vorm van RNA) meestal omgezet tot eiwitten. Deze eiwitten kunnen bijvoorbeeld enzymen zijn die betrokken zijn bij de productie van vluchtige stoffen. Als een gen vaker wordt afgelezen kunnen er meer enzymen worden gemaakt en dus ook meer vluchtige stoffen.

rupsen. Dit laat duidelijk zien dat de signaal-transductie routes betrokken bij verdediging tegen pathogenen en indirecte verdediging tegen rupsen stroomafwaarts van JA en SA verschillen.

Recentelijk is aangetoond dat de *npr1-1* mutant planten minder worden aangevreten door rupsen van de soort *Spodoptera littoralis* vergeleken met wild-type planten. Blijkbaar vermindert NPR1 de resistentie tegen deze rups. Kennelijk zijn de signaaltransductie-routes betrokken bij de indirecte verdediging tegen rupsen van het kleine koolwitje ook verschillend van de signaaltransductie-routes betrokken bij directe verdediging tegen *S. littoralis*. Dit betekent mogelijk dat de signaaltransductie-routes betrokken bij indirect en directe verdediging van *Arabidopsis* tegen rupsen verschillen. Hier moet wel bij worden opgemerkt dat de rupsensoorten ook verschillend zijn. Zoals eerder vermeld zijn rupsen van het kleine koolwitje specialisten die alleen planten eten die glucosinolaten bevatten. *Spodoptera littoralis* daarentegen is een generalist die van vele verschillende plantensoorten kan eten. Dit verschil heeft waarschijnlijk ook invloed op de signaaltransductie-routes die de beide rupsensoorten induceren.

9. Discussie en conclusies

9.1 Discussie

Zowel JA en SA zijn belangrijk in de signaaltransductie-routes betrokken bij verdediging van de plant tegen herbivoren en pathogenen. Of en hoe JA en SA betrokken zijn bij geïnduceerde verdediging hangt af van de aanvaller. Dit houdt in dat JA en SA niet altijd samenwerken. Sterker nog, er zijn veel gevallen bekend waarbij JA en SA elkaar tegenwerken. Dit kan er toe leiden dat een plant zijn verdediging tegen de ene aanvaller versterkt maar daardoor zijn verdediging tegen een andere aanvaller verzwakt. De resultaten beschreven in dit proefschrift tonen aan dat de geïnduceerde indirecte verdediging tegen rupsen van het kleine koolwitje door aantrekking van de sluipwesp *C. rubecula* echter afhankelijk is van zowel JA als SA.

Hoe deze verschillende interacties tussen JA en SA zijn te verklaren is nog niet helemaal duidelijk. Wat wel bekend is, is dat rupsenvraat voornamelijk de concentratie JA in de plant verhoogt, terwijl het bladluizenschade, die vergeleken met rupsen veel minder verwonding van de plant inhoudt, voornamelijk de concentratie SA in de plant verhoogt. Er zijn aanwijzingen dat spintmijten, die de plant meer verwonden dan bladluizen, een meer evenwichtige invloed te hebben op de concentraties SA en JA. Het lijkt waarschijnlijk dat de onderlinge verhouding van de concentraties JA en SA belangrijk is voor de inductie van verschillende verdedigingsmechanismen. Dit zou ook kunnen verklaren waar om *C. rubecula* geuren van rupsbeschadigde *Arabidopsis* planten prefereert over die van bladluisbeschadigde *Arabidopsis* planten. Rupsen induceren dan - via JA en in mindere mate SA - de productie van een ander mengsel geurstoffen door de plant dan bladluizen, die een geurstofmengsel induceren via voornamelijk SA. Spintmijtschade resulteert - via een meer gelijke verhoging van zowel JA en SA concentraties in de plant - in een mengsel geurstoffen dat, vergeleken met het mengsel dat door bladluizen wordt geïnduceerd, meer lijkt op het mengsel geurstoffen van rupsbeschadigde planten. Inderdaad zijn spintbeschadigde *Arabidopsis* planten aantrekkelijker voor *C. rubecula* dan onbeschadigde planten, terwijl bladluisbeschadigde *Arabidopsis* planten dit niet zijn. Alhoewel er duidelijk aanwijzingen zijn voor de invloed van de balans tussen JA en SA op de productie van geurstoffen, moet het definitieve bewijs nog worden geleverd.

Behalve JA en SA zijn waarschijnlijk ook andere plantenhormonen betrokken bij geïnduceerde verdediging. Eén van deze hormonen is 12-oxo-phytodienoic acid (OPDA). Samen met andere gegevens suggereren de resultaten uit hoofdstuk 5 dat OPDA betrokken is bij de geïnduceerde productie van de geurstof TMTT en aantrekking van carnivoren door planten. Een ander hormoon dat een rol zou kunnen spelen is ethyleen. Ethyleen is betrokken bij de inductie van een bepaald verdedigingsmechanisme tegen pathogenen in *Arabidopsis*, verder stimuleert ethyleen de productie van vluchtige stoffen en de aantrekking van carnivore roofmijten door Lima boon en remt het de productie van de toxische stof nicotine in tabak. Of ethyleen ook betrokken is bij de geïnduceerde productie van vluchtige stoffen door *Arabidopsis* is nog onbekend. De rol van zowel OPDA als ethyleen in indirecte verdediging van *Arabidopsis* is dan ook een interessant onderwerp voor vervolgonderzoek.

De geïnduceerde verdediging van de plant tegen de verschillende aanvallers kan niet alleen verschillen in het gebruik van hormonen zoals JA en SA. Ook stroomafwaarts van deze hormonen kunnen de signaaltransductie-routes verschillen (zie paragraaf 8.3). Het is duidelijk dat signaaltransductie-routes betrokken bij geïnduceerde verdediging van planten complex zijn, dat deze afhangen van de soort aanvaller en dat één soort aanvaller via verschillende signaaltransductie-routes verschillende verdedigingsmechanismen kan induceren (bijvoorbeeld directe en indirecte verdediging). Hoe een plant de inductie van al deze verschillende signaaltransductie-routes regelt en hoe deze signaaltransductie-routes elkaar beïnvloeden blijft een intrigerende vraag.

9.2 Conclusies

In dit proefschrift laat ik zien dat *Arabidopsis* een uitstekende modelplant is voor het bestuderen van indirecte verdediging. Door gebruik te maken van de unieke mogelijkheden die *Arabidopsis* biedt heb ik een model opgesteld dat de rol van jasmonzuur en salicylzuur in de door rupsenvraat geïnduceerde productie van vluchtige stoffen beschrijft. De hypotheses die uit dit model voortvloeien kunnen gebruikt worden als leidraad voor vervolgonderzoek. Ook heb ik een begin gemaakt met de ontrafeling van de signalering stroomafwaarts van jasmonzuur en salicylzuur. Hiermee is een belangrijke bijdrage geleverd aan het onderzoek naar indirecte verdediging van planten tegen herbivore insecten en de interacties hiervan met directe verdedigingsmechanismen tegen zowel herbivore insecten als pathogenen.

Curriculum Vitae

Remco Martha Prudent van Poecke was born on a sunny Friday afternoon, June 21st 1974, in the hospital of Oostburg (Zeeuwsch-Vlaanderen, The Netherlands). Although born in Oostburg, he was raised in Sluis, a nearby small border town. He graduated from secondary school in 1992. After enjoying a wonderful childhood in Zeeuwsch-Vlaanderen, he decided that it was time to extend his horizon. Having experienced the delightful atmosphere of Wageningen (Gelderland, The Netherlands) during an introduction day, the choice to study Molecular Sciences at Wageningen Agricultural University (nowadays Wageningen University) was an easy one. He took a major in 'Dynamic expression of transcription factor genes in Petunia'; continued to work on 'Genetic modification of the tryptophan biosynthetic pathway of potato' at the 'Centre for Plant Breeding and Reproduction Research' (CPRO-DLO, nowadays Plant Research International) in Wageningen; and finished his masters degree in 1997 with a practical period in Canada, at the University of Guelph (Ontario, Canada), where he worked on 'The expression of green leaf volatile genes in response to pathogen attack and salicylic acid in *Arabidopsis thaliana*'. In 1998, he started working on a PhD-project at the Laboratory of Entomology of the Wageningen University. The result of this project is now lying before you. Currently, he is still working on *Arabidopsis* and its interactions with insects in a post-doc project at the same lab.

List of Publications

Van der Krol, A.R, Van Poecke, R.M.P., Vorst, O.F.J., Voogt, C., Van Leeuwen, W., Borst-Vrensen, T.W.M., Takatsuji, H., Van der Plas, L.H.W., 1999. Developmental and wound-, cold-, desiccation-, ultraviolet-B-stress-induced modulations in the expression of the petunia zinc finger transcription factor gene *ZPT2-2*. *Plant Physiololgy*, 121: 1153-1162.

Van Poecke, R.M.P., Posthumus, M.A., Dicke, M., 2001. Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: Chemical, behavioral, and gene-expression analysis. *Journal of Chemical Ecology*, 27: 1911-1928.

Dicke, M. and Van Poecke, R.M.P., 2002. Signaling in plant-insect interactions: signal transduction in direct and indirect plant defence. In *Plant Signal Transduction*, Scheel D. and Wasternack C. (eds). Oxford University Press, Oxford, UK, pp.289-316.

Van Poecke, R.M.P. and Dicke, M., 2002. Induced parasitoid attraction by *Arabidopsis thaliana*: Involvement of the octadecanoid and the salicylic acid pathway. *Journal of Experimental Botanty*, 53: 1793-1799.

Van Poecke, R.M.P., Posthumus, M.A., Dicke, M., to be submitted. Signal transduction in induced volatile emission by *Arabidopsis*: molecular genetic and chemical analysis of different genotypes.

Van Poecke, R.M.P. and Dicke, M., to be submitted. Signal transduction downstream of salicylic and jasmonic acid in herbivory-induced parasitoid attraction by *Arabidopsis*.

Van Poecke, R.M.P., Roosjen, M., Pumarino, L., and Dicke, M., to be submitted. Attraction of the specialist parasitoid *Cotesia rubecula* to *Arabidopsis thaliana* infested by host or non-host herbivore species.

Printed by Ponsen & Looijen bv, Wageningen, The Netherlands