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Arabidopsis-Insect Interactions

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1. INTRODUCTION

1.1 Plant-Insect Interactions

Insects are the most species-rich class of eukaryotes on earth and roughly half of all insect species are herbivores. Besides being species-rich, insects are also quite abundant, making up more biomass than any other animal class. It is therefore likely that during their life, most plants will encounter herbivorous insects chewing on their tissue, sucking up their cell content and/or passively feeding on their vascular sap. Not surprisingly, plants are not defenseless against these herbivores. Plants have evolved a wide range of defense mechanisms which can be constitutively present and/or induced by herbivory. Many of these defense mechanisms have a direct effect on the herbivore by negatively affecting its physiology (e.g. through toxins or anti-nutritional compounds) or by interfering with its behavior (e.g. through repelling or deterring compounds) (Schoonhoven et al., 1998). Besides direct defense mechanisms, plants can make use of 'bodyguards' to protect themselves against herbivorous insects. Often, these bodyguards are insects themselves: predators or parasitoids preying or parasitizing on the herbivores. Plants can reward these bodyguards by providing them shelter, additional food and/or information (Van Poecke and Dicke, 2004). But plants do not only rely on insects as bodyguards. For many plant species insects form an essential part of plant reproduction by transporting their pollen (Pichersky and Gershenzon, 2002). Clearly, insects play an important part in the ecology of plants, whether considered from a natural, agricultural or an evolutionary perspective. In the last decade, Arabidopsis thaliana has been introduced to studies on plant-insect interactions, especially with the aim to get a better understanding of the plant molecular mechanisms underlying this interaction. As the number of publications on Arabidopsis - insect interactions is steadily increasing, this review aims to summarize their most important findings.

1.2 Arabidopsis-Insect Interactions

A major critique on the use of Arabidopsis as a model plant for plant-insect interaction is that, as a winter annual, the life cycle of Arabidopsis does not temporally overlap with the life cycle of many herbivorous insects. Indeed, in a study on the effect of life-cycle strategies of crucifers on herbivory, Yano and Ohsaki (1993) used Arabidopsis to demonstrate that plants with a 'pausing' strategy (i.e. not having any above ground alive tissue in summer) dramatically reduced herbivore presence, both in numbers and in species. Additionally, they found that herbivore performance (measured as mortality and developmental time) was better on plants with a 'pausing' strategy, indicating that such plants may invest less in defense traits. However, not all Arabidopsis have a winter annual life style (Pigliucci, 2002), and herbivores, mainly flea beetles but also aphids, leaf miners and caterpillars have been reported on Arabidopsis in the field (Mauricio, 1998; Geervliet, 1997). Note that table 1 contains a list of all insect species mentioned in this chapter.

In the lab, most researchers working with Arabidopsis must have seen Arabidopsis-insect interactions regularly when combating fungus gnats or aphids. Indeed, one of the first papers about Arabidopsis-insect interactions dealt with the fungus gnat *Bradysia impatiens* (McConn et al., 1997) and since then, many other chewers, piercers and suckers that dine on Arabidopsis have been studied (figure 1; see table 1 for a list of insects mentioned in this chapter). From these studies it becomes clear that Arabidopsis is far from defenseless against arthropod attackers. For example, many Arabidopsis accessions have leaves that are quite densely covered with trichomes, known to hamper moths and flea beetles, and the leaves contain toxins and feeding deterrents such as glucosinolates and proteinase inhibitors that are known to be effective against many herbivores (see Handley et al., 2005; Farmer and Ryan, 1990; Louda and Mole, 1991; and Part II). Additionally, Arabidopsis emits volatiles upon herbivory that can attract natural enemies of the herbivores, such as parasitoids; and volatiles emitted by its flowers may even

Table 1. Insect species mentioned in this chapter

Latin Name	Common name	Order	Common classification
Generalists[1]			
Bradysia impatiens	Fungus gnat	Diptera	Gnat
Franklineilla occidentalis	Western Flower Thrips	Thysanoptera	Thrips
Helicoverpa sp.[2]		Lepidoptera	Moth
Mamestra brassicae	Cabbage moth	Lepidoptera	Moth
Myzus persicae	Peach aphid	Hemiptera	Aphid
Phyllotreta nemorum	Striped flea beetle	Coleoptera	Flea beetle
Spodoptera exigua.	Beet armyworm	Lepidoptera	Moth
Spodoptera frugiperda	Fall armyworm	Lepidoptera	Moth
Spodoptera littoralis	Egyptian cotton worm	Lepidoptera	Moth
Spodoptera litura[2]	Cluster caterpillar	Lepidoptera	Moth
Trichoplusia ni	Cabbage looper	Lepidoptera	Moth
Specialists[1]			
Brevicoryne brassicae	Cabbage aphid	Hemiptera	Aphid
Chrysomela populi[2]	Poplar leaf beatle	Coleoptera	Leaf beetle
Lipaphis erysimi[2]	Turnip aphid	Hemiptera	Aphid
Pieris brassicae	Large cabbage white	Lepidoptera	Butterfly
Pieris rapae	Small cabbage white	Lepidoptera	Butterfly
Plutella xylostella	Diamondback moth	Lepidoptera	Moth
Others			
Cotesia rubecula		Hymenoptera	Parasitoid

[1]Note that the distinction generalist - specialist is rather arbitrary
[2]These insects are mentioned in the text, but not with respect to interactions with Arabidopsis

attract pollinators (Van Poecke, et al., 2001; Chen et al., 2003b). Thus, Arabidopsis-insect interactions may be of relevance in the field and even if certain interactions are unlikely to pose evolutionary selective pressures, studying them can still yield valuable information on mechanisms behind traits that may be more important for other plant species. For example, indirect defense of Arabidopsis by attracting the parasitoid *Cotesia rubecula* may not be of importance for this plant. Still, Arabidopsis can attract these parasitoids and many aspects of this trait are similar in Arabidopsis compared to other plant species (Van Poecke and Dicke, 2004). Thus, Arabidopsis is a valuable model plant for studying plant-insect interactions, as is demonstrated by the work summarized in the following sections.

2. MORPHOLOGICAL AND BIOCHEMICAL DEFENSES

Most studies on Arabidopsis-insect interactions deal with defense against herbivores. Many of the plant defenses, such as trichomes, glucosinolates and proteinase inhibitors are constitutively present. These constitutive levels of defenses form a basal line of defense, which can be strengthened upon perception of herbivory by inducible defenses. Inducible defenses include both morphological and biochemical defenses and may include mechanisms that are already constitutively present (Karban and Baldwin, 1997). The genetic variation and sequence information available for Arabidopsis has had an impressive impact on our understanding of plant defenses in general and the biosynthesis defense structures and chemicals in particular. In this part, I will describe the biosynthesis/development and functionality of Arabidopsis defense mechanisms against insects.

2.1 Trichomes

Trichomes are epidermal projections that come in all kinds of shapes and sizes and can be found on all aerial plant parts, although plant species, accessions and cultivars differ in the presence of trichomes on the different organs. According to some definitions, root hairs form a distinct class of trichomes and indeed there are striking similarities in the genetics of root hair and (aerial) trichome formation (Werker, 2000; Serna, 2004). Aerial trichomes can be divided into two general types: glandular and non-glandular. The functions of trichomes, partly dependent on type, are plentiful, ranging from light reflectance, to climbing assistance (as in cleavers) to, of course, herbivore resistance (reviewed by Jeffree, 1986 and Wagner et al., 2004). Arabidopsis trichomes have been studied mainly in relation to cell-fate initiation and development, although a few

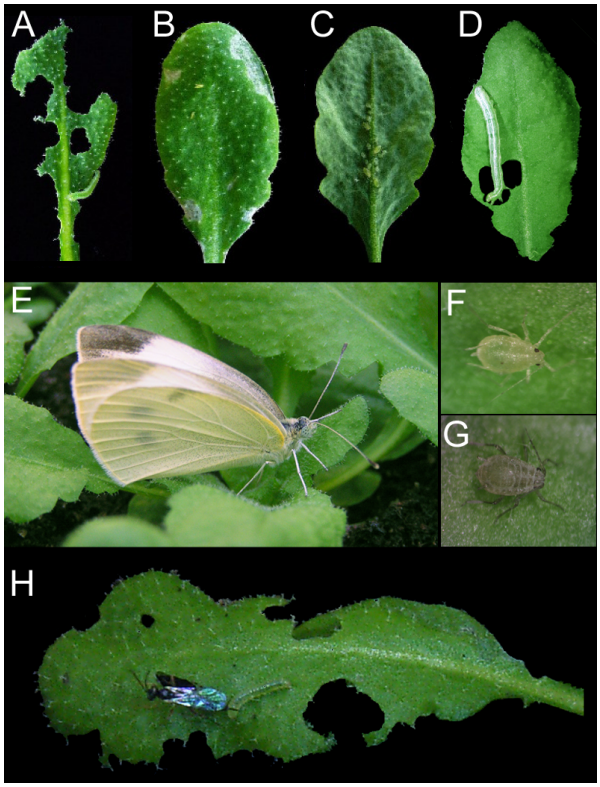

Figure 1. (A) Leaf damage by early instar *P. rapae* caterpillars; (B) Leaf damage by *Franliniella occidentalis* thrips larvae; (C) *Myzus persicae* aphids accumulating near the mid-vein; (D) Leaf damage by late instar *Trichoplusia ni* caterpillar; (E) *P. rapae* female butterfly preparing to oviposit; (F) Close-up of *M. persicae* on Arabidopsis; (G) Close-up of *Lipaphis erysimi* aphid on Arabidopsis; (H) *Cotesia rubecula* female parasitoid after ovipositing on its host *P. rapae.* (A)-(C) adapted with permission from De Vos et al., (2005); (D)-(G) by Jetske De Boer & Remco Van Poecke; (H) by Tjeerd Snoeren.

studies have looked at the effect of Arabidopsis trichomes on insect herbivores.

2.1.1 Development

Arabidopsis trichomes are non-glandular, single-cell structures originating from epidermal cells and are found mainly on the adaxial surface of the leaves, but also on the abaxial leaf surface and on stems and sepals, with stem and sepal trichomes usually being unbranched and leaf trichomes having two to four branches depending on accession (Hulskamp and Kirik, 2000). Initiation of trichome formation in Arabidopsis starts and finishes in the leaf primordium stage, with the first trichome forming from an epidermal cell at the tip of the leaf when the primordia are about 100 um long and with trichome initiation being completed when primordia are about 700 um long in the accession Col-0 (Larkin et al., 1996). There is substantial variation between Arabidopsis accessions in trichome density (Mauricio, 2005; Symonds et al., 2005), ranging from completely hairless accessions (such as Mir-0 and Kas-1) to densely covered accessions such as Chi-1 (figure 2). Excluding hairless plants, differences between accessions in trichome density may be reflected in the duration of trichome initiation. For example, trichome initiation in L*er* stops when the primordia are about 500 um long and L*er* is less-densely covered with trichomes than Col-0 (Larkin et al., 1996). The genetic background of both trichome initiation and development is reasonably well understood, with a large array of mutants available (reviewed by Hulskamp and Kirik, 2000; Marks and Esch, 2003; Szymanski, 2005).

2.1.2 Plant-Insect Interactions

For arthropod herbivores, trichomes can be both a blessing and a curse. As structural features, they may hinder or help landing, impede movement, alter the microclimate, reduce predation rates when they hamper carnivores and can be climbed by small herbivores to avoid detection by predators. On the other hand, trichomes may increase predation rates as some predators lay their eggs in trichome dense areas to avoid intra-guild predation and can use pollen trapped by trichomes as alternative food source (Southwood, 1986; Krips et al., 1999; Roda et al., 2000; Michalska, 2003; Roda et al., 2003; Stavrinides and Skirvin, 2003). In the case of glandular trichomes, they are also part of chemical defenses against herbivores (e.g Chatzivasileiadis and Sabelis, 1997; Maluf et al., 2001), but as trichomes in Arabidopsis are non-glandular, single cells I will not discuss this further. So far, there is no evidence that defense compounds accumulate in Arabidopsis trichomes. Identification of 63 proteins from trichomes revealed no proteins with known defense functions (Wienkoop et al., 2004). On the other hand, some genes with a possible function in plant defense against insects, such as AtBSMT1 involved in benzoid and salicylic acid methylation, are highly expressed at the base of trichomes

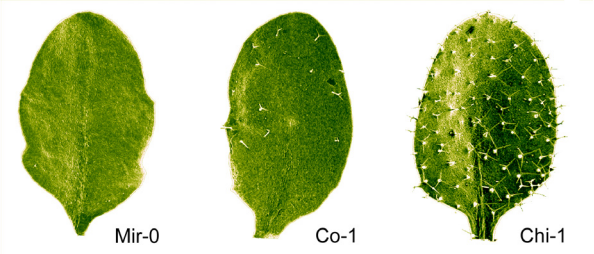

Figure 2. Arabidopsis leaf trichomes. Three Arabidopsis accession showing difference in leaf trichome densitiy. Figure adapted with permission from Symonds et al. (2005). Color on the original black and white picture was added using Adobe Photoshop 8.0.

(Chen et al., 2003a; Wang et al., 2005). Indeed, there are some indications that trichomes are involved in volatile emissions and as such play a role in indirect defense (see §3.21.4.2).

Several studies provide evidence for a role of trichomes in defense of Arabidopsis against herbivores. Reymond et al. (2004) showed that *Pieris rapae* caterpillars gained more weight on glabrous (=hairless) *gl1* mutants compared to wild-type Columbia. Additionally, correlation between trichome density and plant defense was demonstrated in two studies on Arabidopsis populations: In a study using plants collected from various fields in Sweden, Handley et al. (2005) demonstrated that oviposition by *Plutella xylostella* moths was negatively correlated with trichome density. However, no correlation was found between performance of *P. xylostella* larvae and trichome density. In a field study in North Carolina, Mauricio (1998) found a negative correlation between feeding damage (likely done for the most part by flea beetles) and trichome density. The latter study also demonstrated that defenses such as trichomes may be costly: in undamaged plants, trichome density was negatively correlated with fruit production, with costs exceeding the benefits in herbivore-damaged plots (see also §4.1.2).

2.1.3 Inducibility

Presumably to minimize costs, plant defenses are often inducible. As trichome density is determined early in leaf development (Mauricio, 2005), a damaged leaf will not start to grow extra trichomes. Nonetheless, wounding of Arabidopsis plants does lead to increased trichome density of newly formed leaves, although this has not been demonstrated as a response to herbivory (Traw and Bergelson, 2003).

2.2 Epicuticular Waxes

Together with trichomes, epicuticular waxes are the first line of defense that a herbivore encounters upon contact with a plant. Waxes can vary from being spiky due to crystal formation, as seen on the stem and siliques of Arabidopsis, to very smooth, as seen on Arabidopsis leaves (Jenks et al., 2002; figure 3). The chemical constitution and physical properties (such as thickness of the wax layer) of epicuticular waxes are known to influence both herbivore oviposition and feeding (Eigenbrode and Espelie, 1995). For example, defense compounds such as glucosinolates (see below) have been detected in leaf waxes of *Brassica oleracea* (van Loon et al., 1992), but no such compounds have been reported for Arabidopsis epicuticular wax (Rashotte et al., 2004). Even though many Arabidopsis mutants and accessions with differences in epicuticular waxes are known (see Koornneef et al., 1989; Rashotte et al., 1997; Rashotte et al., 2004), very little has been published on the effect of these different genotypes on herbivore behavior and performance. In this book, Jenks et al., (2002) reported differences in egg-laying behavior of the moth *P. xylostella* on different wax mutants and their extracted waxes. More recently, the behavior of neonates and development time of the larvae of *P. xylostella* on different wax-mutants and their waxes was studied, showing differences in neonate behavior (such as time to first feeding and biting duration) but no difference in development time (J.J.A. van Loon, personal communication). The chapter by Jenks et al. (2002) also reported effects of epicuticular waxes on aphid behavior and performance, with *Brevicoryne brassicae* probing less and walking more on a particular wax-mutant (*cer-3*), which was correlated with a lower aphid fecundity on this mutant compared to wild-type plants. As these wax mutants mainly differ in their composition of primary alcohols in their waxes, these results suggest roles for primary alcohols in plant-insect interactions.

2.3 Glucosinolates

Glucosinolates are secondary plant metabolites found in plants belonging to the order Capparales, including families like capers (Capparaceae) and crucifers (a.k.a. mustards, or Brassicaceae). They are mainly known as the reason why kids hate Brussels sprouts, but also many generalist herbivores (herbivores that feed on many plant species) dislike glucosinolates (Wittstock et al., 2003). Indeed it appears that glucosinolate-containing plants suffer less from insect herbivory than plants without glucosinolates (Louda and Mole, 1991). However, many herbivorous insect species have overcome glucosinolate-defenses and use them as oviposition and feeding stimulants, thus specializing on glucosinolate-containing plants

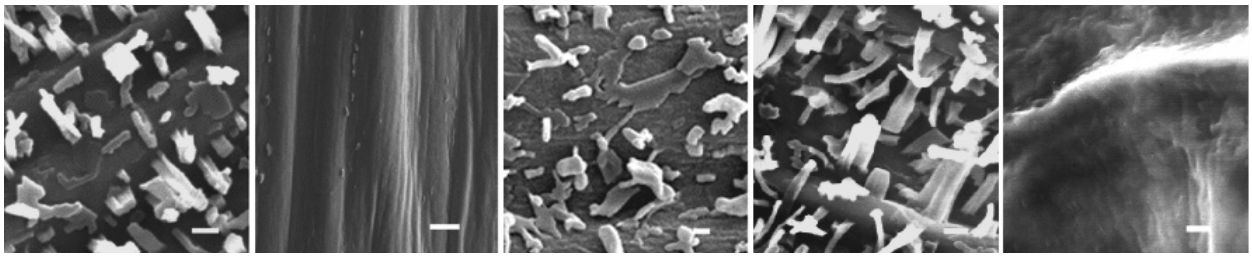

Figure 3. Arabidopsis waxes. Scanning electron microscope images of waxes from (from left to right): flowering stems from accession L*er*, mutants *cer6* and *cer15* (both in L*er* background), accession Ws and abaxial leaf from Ws. Figure adapted with permission from Jenks et al. (2002).

(Schoonhoven et al., 1998). Glucosinolate-degradation products are also known to attract natural enemies of glucosinolate-adapted herbivores (Louda and Mole, 1991; Schoonhoven et al., 1998). Because of these interesting interactions with insects, glucosinolate-containing plants such as crucifers have been used in ecological and evolutionary studies for decades. The number of publications on glucosinolates using Arabidopsis has expanded dramatically in the last couple of years.

2.3.1 Biosynthesis

Synthesis, degradation and function of glucosinolates have been reviewed recently (Wittstock and Halkier, 2002; Kliebenstein, 2004); the following is a short overview. Glucosinolates are amino acid-derived metabolites containing a sulphate and a thioglucose moiety. Depending on the amino acid from which they are derived, glucosinolates can be divided into three classes: aliphatic glucosinolates (in Arabidopsis derived from methionine and some from leucine), aromatic glucosinolates (in Arabidopsis derived from phenylalanine) and indole glucosinolates (derived from tryptophan) (figure 4A). In total, 36 Arabidopsis glucosinolates have been found using various accessions (Reichelt et al., 2002). Additionally, glucosinolate profiles were studied at different developmental stages in all major organs in Col-0 (Brown et al., 2003). Together, these studies reveal that: methionine-derived aliphatic glucosinolates are dominant in all organs at almost all developmental stages; during development, early leaves contain less glucosinolates than later leaves; reproductive organs and especially seeds contain the highest levels of total glucosinolates; seeds also contain the highest diversity of glucosinolates, with aromatic glucosinolates being almost exclusively present in seeds. Between accessions, seeds mainly differ in indole glucosinolate composition and leaves mainly show variation in aliphatic glucosinolate composition. The variation in glucosinolate profiles between accessions may be based on relatively few genes as polymorphism at five loci was sufficient to generate 14 qualitatively different glucosinolate profiles (figure 4B). The dramatic impact of relatively few genes on glucosinolate profiles constitutes an evolutionary flexible system that may be easily adapted to different selective pressures (Kliebenstein et al., 2001b; and see §4.2.1).

2.3.2 Degradation

Although glucosinolates may function as storage compounds for sulphur and nitrogen, their most important role is likely in plant defense. However, not the glucosinolates themselves but rather their degradation products are most active in defense against both herbivores and pathogens (figure 4C). Similar to many other compounds, the glucoside form likely functions as an inactive precursor (Rask et al., 2000). The first step in glucosinolate degradation is the removal of glucose, catalyzed by myrosinase. Myrosinases are compartmentalized in myrosin bodies of specialized myrosin cells, which in Arabidopsis are confined in phloem parenchyma (Andreasson et al., 2001). Glucosinolates can be found in the vacuoles of cells in all plant organs but in the main flower stalk are particularly concentrated in special cell types lining the phloem (Koroleva et al., 2000). Upon tissue-disruption, myrosinases and glucosinolates come into contact, triggering the degradation of glucosinolates. A second step, after removal of glucose, further degrades the glucosinolates to their final products. Arabidopsis accessions can be divided into two classes with respect to the type of final products: those that predominantly produce nitriles and those that predominantly produce isothiocyanates. Accessions having a functional epithiospecifier protein (ESP), such as L*er*-0 and Cvi-0 produce nitriles or epithionitriles from alkyl or alkenyl glucosinolates respectively; accessions with a non-functional ESP, such as Col-0 and Ws-0 , produce isothiocyanates (Lambrix et al., 2001; Zabala et al., 2005). Recently, another gene involved in glucosinolate degradation has been cloned. This protein, epthiospecifier modifier1 (ESM1), drives glucosinolate degradation towards isothiocyanates. Functional activity of both ESP and ESM1 result in a mixture of isothiocyanates and nitriles (Zhang et al., 2006).

2.3.3 Plant-Herbivore Interactions

That glucosinolate degradation products can be toxic to insects has been demonstrated in many studies, with isothiocyanates being especially toxic (reviewed by Wittstock et al., 2003; and see Lambrix et al., 2001; Rohloff and Bones, 2005 for a list of glucosinolate-degradation products found in Arabidopsis). The mode of action of this toxicity is still unclear, although isothiocyanates are known to react with proteins. Additionally, both nitriles and isothiocyanates may impact cellular respiration through HCN production and other mechanisms (Tsao et al., 2002; Wittstock et al., 2003).

There are several examples of negative correlation between Arabidopsis glucosinolate levels and herbivore performance: (Mauricio, 1998) showed a negative correlation between glucosinolate levels in Arabidopsis and herbivore damage in the same North Carolina field study mentioned before (§ 3.2). Kroymann et al. (2003) demonstrated that the L*er MAM2* gene is responsible for enhanced aliphatic glucosinolate levels and reduced feeding damage by larvae of the generalist moth *Spodoptera exigua*; Kliebenstein et al. (2005) reported a negative correlation between aliphatic glucosinolate levels and herbivore damaged caused by larvae from *S. exigua* and another generalist moth, *Trychoplusia ni*; and Mewis et al., (2005) showed that performance of *S. exigua* and the specialist aphid *B. brassicae* and generalist aphid *Myzus persicae* was negatively correlated with total glucosinolate levels. Additionally, Mauricio (1998) showed a positive correlation between glucosinolate levels and plant fitness in herbivore damaged plants, measured as the number of siliques produced. From these results, it becomes clear that glucosinolates act as defense compounds in interactions between Arabidopsis and herbivores.

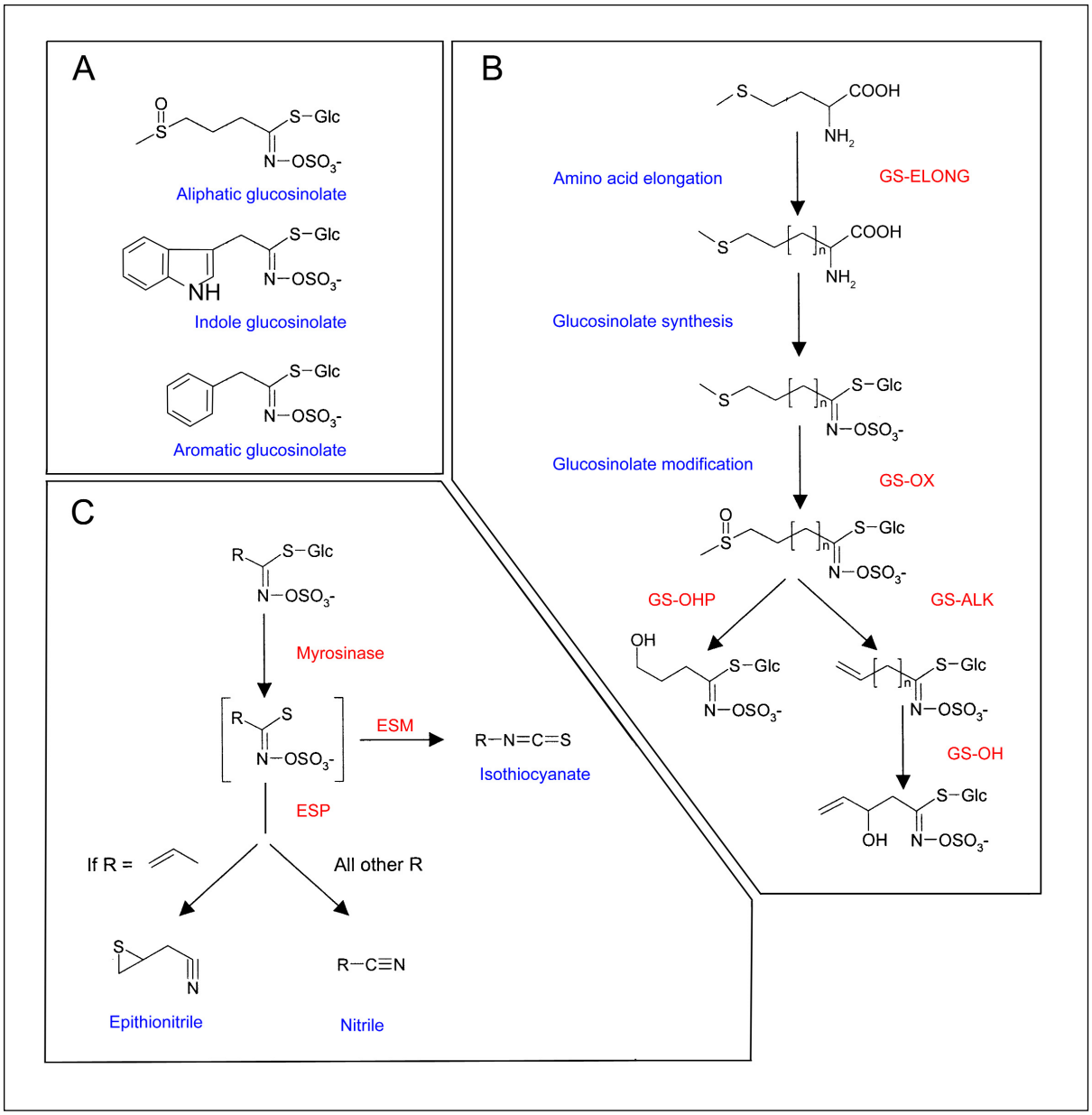

Figure 4. Glucosinolates. **(A)** Examples of the three classes of glucosinolates: aliphatic; indole; and aromatic glucosinolates, here represented by 3-methylsulfinylpropyl glucosinolate; indol-3-ylmethyl glucosinolate; and benzyl glucosinolate. **(B)** Simplified biosynthesis of glucosinolates in three steps, illustrated here with the biosynthesis of methionine-derived glucosinolates, the largest and most diverse group of glucosinolates in Arabidopsis. The three steps are indicated in blue: amino acid elongation (optional); glucosinolate synthesis; and glucosinolate modification (optional). The optional steps are not required for every glucosinolate. Five major loci controlling much of the variation in glucosinolate composition are indicated in red. GS-ELONG is involved in side-chain elongation (n indicates number of elongation cycles); GS-OX converts methylthioalkyl glucosinolates to methylsulfinylalkylglucosinolates; GS-OHP and GS-ALK represent two different alleles from the GS-AOP locus, GS-OHP only acts on 3 carbon side-chain glucosinolates (n=1) and produces hydroxypropyl glucosinolates and GS-AOP produces alkenyl glucosinolates; GS-OH hydroxylates alkenyl glucosinolates and only acts on 4 carbon side-chain glucosinolates. **(C)** Degradation of glucosinolates leading either to isothiocyanate or nitrile production depending on activity of ethiospecifier protein (ESP) and/or ethiospecifier modifier1 (ESM1). Enzyme activities are indicated in red. Figure adapted with permission from Kliebenstein, et al. (2004).

However, larvae from the specialist moth *P. xylostella* do not seem to suffer from the elevated aliphatic glucosinolate levels associated with *MAM2* expression. On the contrary, damage by this herbivore was positively correlated with aliphatic glucosinolate levels and herbivore damaged caused by the specialist *P. xylostella* (Kroymann et al., 2003; Kliebenstein et al., 2005; see also §4.2.1). These examples show that even though glucosinolates are considered defense compounds, several herbivore species have been able to overcome this defense system. Once herbivore species can overcome plant defenses they may specialize on plant species containing these defenses. Ratzka et al. (2002) discovered that larvae of the crucifer specialist *P. xylostella* can detoxify glucosinolates by desulfating them, with sulfatase activity and gene-expression only found in the gut of the larvae. Other crucifer specialists such as *P. rapae* and *Pieris brassicae* caterpillars do not show sulfatase activity, indicating that there must be more strategies to cope with glucosinolates. Indeed it was recently demonstrated that a protein in the gut of *P. rapae,* a designated nitrile-specifier protein, diverts of degradation of glucosinolates from isothiocyanates to nitriles, when feeding on an accession (Col-0) that normally produces isothiocyanates (Wittstock et al., 2004). This fits well with the observation that *P. rapae*-infested Col-0 plants emit nitriles and not isothiocyanates (Van Poecke et al., 2001).

When herbivores can overcome plant defenses, they may use these mechanisms to their own advantages. For example, specialist herbivores can use the glucosinolates and/or their degradation products as volatile attractants, and feeding and oviposition stimuli (see review by Rask et al., 2000). Indeed some glucosinolate degradation products can be found in trace amounts in the headspace of undamaged or caterpillar-infested plants (Van Poecke et al., 2001; Vercammen et al., 2001; Rohloff and Bones, 2005). Specialized insects may even sequester glucosinolates for their own defense against predators (Muller and Wittstock, 2005). An intriguing example comes from the specialist aphids *B. brassicae* and *Lipaphis erysimi*, that besides sequestering glucosinolates, have myrosinases confined to specialized microbodies, similar to the situation in plants. Possibly, they function as a defense against predators in a similar fashion to plants, such that tissue disruption results in the production of isothiocyanates, that may not only be toxic to predators but additionally act as synergists to the aphid alarm pheromone E-beta-farnesene (Bridges et al., 2002).

In short, glucosinolates function as defense compounds against generalist herbivores but may be exploited by specialist herbivores. This difference between specialist and generalist herbivores was reflected in QTL analyses performed by Kliebenstein et al. (2001a), where QTL analyses of resistance against generalist *T. ni* caterpillars resulted in six loci, of which five were involved in either glucosinolate biosynthesis or breakdown (one of these loci corresponds to the ESP locus [Lambrix et al., 2001], which may be identical to the TASTY locus found in a different QTL study on plant resistance using *T. ni* and L*er* x Col recombinant inbred lines [Jander et al., 2001]). In contrast, neither of two QTL affecting resistance against the specialist *P. xylostella* caterpillars overlapped with glucosinolate biosynthesis or degradation QTL. Besides different effects of glucosinolates on generalists and specialists, these results also indicate that 1) aliphatic glucosinolates deter *T. ni* herbivory, 2) increased myrosinase activity decreases *T. ni* herbivory and 3) *T. ni* prefers nitriles over isothiocyanates (the latter was also demonstrated by Lambrix et al., 2001). An intriguing question arises from these studies: if isothiocyanates are more effective as defense compounds against generalist herbivores, why do some accessions form nitriles instead of isothiocyanates, which requires an additional enzymatic step? One hypothesis is that as isothiocyanates also function as oviposition stimuli for specialist herbivores, it may be beneficial not to produce these compounds when selection pressure by specialists is high. Another hypothesis is that nitriles may be more effective against generalist herbivores other than *T. ni* (Lambrix et al., 2001). With respect to the latter, it should be noted that although *T. ni* is a generalist herbivore, cruciferous plants are among its preferred hosts, hence the common name cabbage looper (Soo Hoo et al., 1984).

2.3.4 Tritrophic Interactions

Besides influencing plant-herbivore interactions directly, glucosinolates also may be important to a third trophic level. Predators or parasitoids that specialize on crucifer feeding herbivores can use volatile glucosinolate degradation products as cues to locate their prey or host. For example, the profile of isothiocyanates produced by different *Brassica oleracea* near-isogenic lines influences parasitoid behaviour (Bradburne and Mithen, 2000). Van Poecke et al. (2001) demonstrated that the parasitoid wasp *C. rubecula* distinguishes between mechanically damaged Col-0 and Col-0 infested by it's host *P. rapae*. One of the differences in the volatile blends of these odor sources was the presence of nitriles in the blend of *P. rapae*-infested plants. Thus, it may be that *C. rubecula* wasps use nitriles to locate their host. However, this parasitoid did not distinguish between *P. xylostella*-infested plants and *P. rapae* infested plants (Van Poecke et al., 2003). As *P. xylostella* desulfates glucosinolates (Ratzka et al., 2002) rather than diverting degradation to nitriles, it appears that althought *C. rubecula* may use nitriles for host-location, it does not use these nitriles for host-discrimination.

2.3.5 Inducibility

The same field study that showed that total glucosinolate level is negatively correlated with feeding damage in natural populations of Arabidopsis, also showed that for undamaged plants, glucosinolate level was negatively correlated with fruit production (Mauricio and Rausher, 1997; Mauricio, 1998). Additionally, undamaged mutant plants that produced less glucosinolates, also showed increased fitness compared to undamaged wild-type plants

(Mauricio, 2001). This suggests that there are costs related to glucosinolate defenses. Indeed, glucosinolate levels - especially aliphatic glucosinolates - can be induced by caterpillar and aphid feeding (Mewis et al., 2005), possibly to minimize these costs.

2.4 Terpenoids

Terpenoids comprise the most diverse group of plant metabolites and have both primary and secondary functions (Schoonhoven et al., 1998). Primary functions include hormone signaling (gibberellins and brassinosteroids) and photosynthesis (carotenoids and side-chains of chlorophylls). Secondary functions include plant-plant communication, defense against pathogens and plant-insect communication (Aubourg et al., 2002). Terpenoids make roses smell nice, not only to our noses but likely also to pollinators (Langenheim, 1994; Antonelli et al., 1997). Terpenoids also form the characteristic smell of pine trees that repels many herbivorous insects (Gershenzon and Croteau, 1991). Although not famous for being odoriferous, Arabidopsis plants do emit terpenoids.

2.4.1 Biosynthesis

Terpenoids are produced in the plant through two distinct pathways: the mevalonate (MVA) pathway is located in the cytosol/ endoplasmatic reticulum and produces sesqui- and triterpenoids, the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is located in plastids and produces mono-, di- and tetraterpenoids (figure 5). The pathways converge biochemically in the production of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). IPP and DMAPP form the building blocks of terpenoids and are linked through the action of prenyltransferases. The products of prenyltranferases are used by terpene synthases (TPS) to produce primary and secondary metabolites (Aubourg et al., 2002; Rodriguez-Concepcion and Boronat, 2002). In Arabidopsis 40 putative TPS genes have been identified of which 32 are apparently intact. Most of these are predicted to be involved in secondary metabolite production (Aubourg et al., 2002). Characterized genes include five monoterpene synthases and two sesquiterpene synthases (Bohlmann et al., 2000; Chen et al., 2003b; Fäldt et al., 2003; Chen et al., 2004; Tholl et al., 2005).

Expression analyses showed that the most diverse array of TPS gene-expression is found in flowers, including two of the five characterized monoterpene synthases and the two characterized sesquiterpene synthases. Of the other three characterized monoterpene synthases, two are expressed throughout the plant and one is almost exclusively expressed in roots (Chen et al., 2003b).

Volatile emissions reflect gene-expression in both quantity and quality, with expression of characterized TPS genes correlating nicely with the terpenoids found: flowers emit the highest amount and diversity of terpenoids, very limited amounts of terpenoids are emitted by the vegetative tissue and only one terpenoid could be detected in root-exudates. (Van Poecke et al., 2001; Aharoni et al., 2003; Chen et al., 2003b; Chen et al., 2004; Steeghs et al., 2004; Tholl et al., 2005). Flowering Arabidopsis show a diurnal rhythm of terpenoid emissions, with a peak in emissions during the day; for non-terpenoid volatiles, no such rhythm could be found (Aharoni et al., 2003). Analyses of the emission of selected mono- and sesquiterpenes from flowers from 37 Arabidopsis accessions showed mainly quantitative and a few qualitative differences (Tholl et al., 2005).

While examining transgenic plants overproducing the monoterpene linalool, Aharoni et al. observed that much of the linalool in these plants was glycosylated and glycosylated linalool could also be detected in wild-type plants, albeit in lesser amounts. This indicates that a significant fraction of the terpenoid pool may be stored in a biologically inactive form.

2.4.2 Plant-Insect Interactions

Terpenoids are known to influence herbivorous insects in various ways: they can be part of direct defenses as repellents, feeding deterrents or toxins, with the toxicity of terpenoids possibly due to neurotoxic effects (Garcia et al., 2005; reviewed by Gershenzon and Croteau, 1991 and Langenheim, 1994). Terpenoids can also be used as attractants and/or feeding or oviposition stimulants by specialist herbivores; they can be part of indirect defense as attractants for carnivores; and they can be part of sexual reproduction and outcrossing as attractants of pollinators (Gershenzon and Croteau, 1991; Langenheim, 1994).

With respect to the latter, it is interesting to note that, although Arabidopsis is a self-pollinating species, Arabidopsis flowers emit by far the largest amount of volatiles, especially terpenoids, compared to other plant parts, with some terpene synthase genes being exclusively expressed in flowers. Based on this result and the fact that in Arabidopsis 1) a low percentage of cross-pollination occurs under natural conditions, 2) the receptive stigma protrudes out of the petals before the stamen are mature, 3) floral nectarines are present at the base of the stamen, and 4) the flowers are visited by small insects, Chen et al. (2003b) argued that pollinators may be involved in Arabidopsis outcrossing. The diurnal rhythm found by Aharoni et al. (2003) would suggest that these pollinators are mainly day-active. However, as terpenoids also readily react with reactive oxygen species and can have antimicrobial properties, it is also possible that floral terpenoids protect the reproductive organs from oxidative stress and pathogens (Chen et al., 2003b).

Undamaged vegetative parts of Arabidopsis do not emit many volatiles, but wounding and herbivory by caterpillars result in an increased emission of volatiles, including terpenoids (Van Poecke et al., 2001; Fäldt et al., 2003). As the increase in terpenoid emissions coincides with increased attraction of parasitoid wasps, terpenoids may

be involved in indirect defense of Arabidopsis (Van Poecke et al., 2001). Indeed mutant plants that show reduced emission of terpenoids also showed reduced attraction of parasitoid wasps. However, the role of terpenoids cannot be deduced from this study, as these plants also emit less methyl-salicylate, another known attractant of carnivores (Van Poecke, 2002; Van Poecke and Dicke, 2002; De Boer and Dicke, 2004).

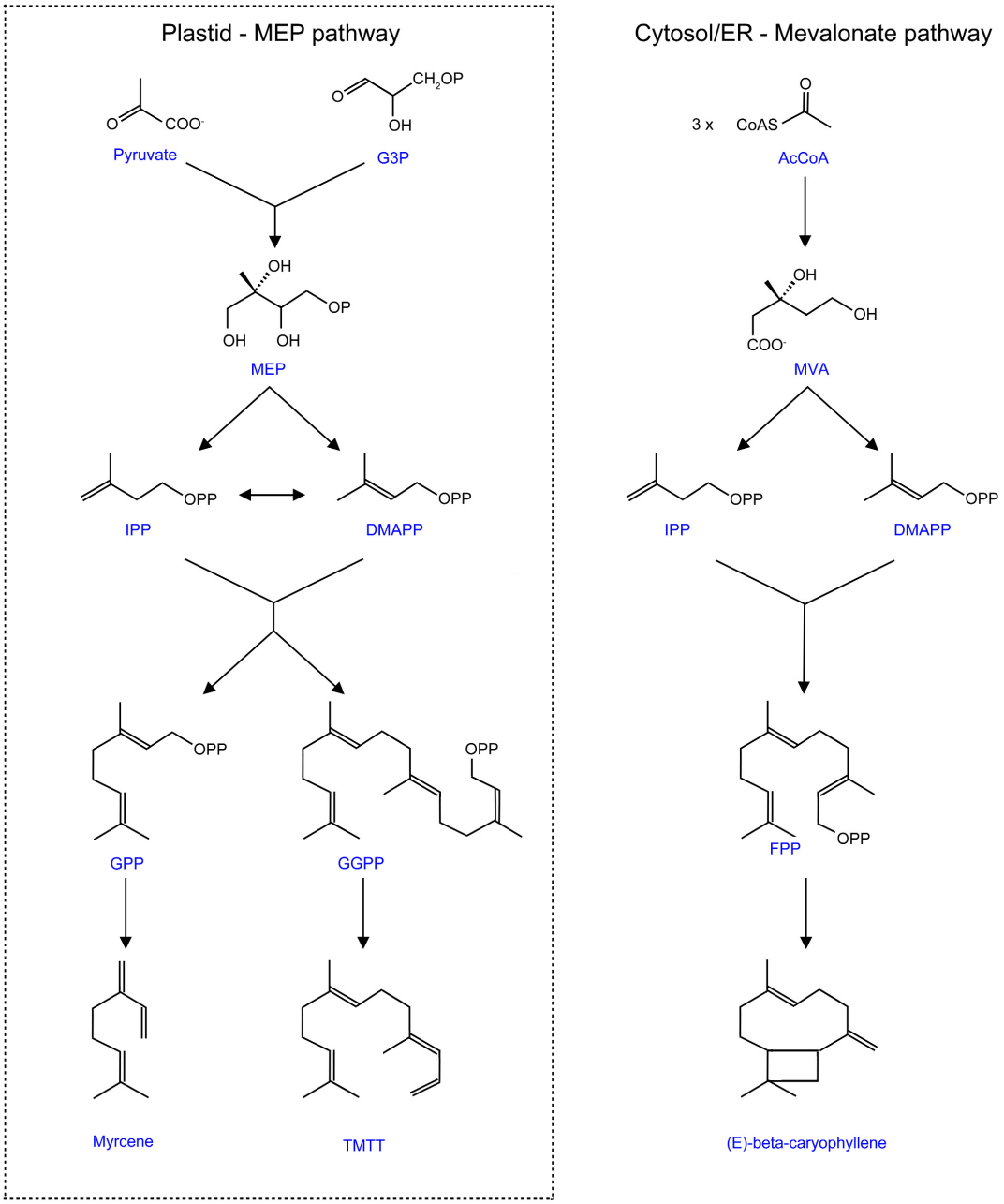

Figure 5. Terpenoid biosynthesis. Biosynthesis of mono- and di-terpenes via the plastidal MEP pathway and of sesquiterpenes via the cytosolic mevalonate pathway for three compounds commonly found in Arabidopsis. Compound names in blue. For abbreviations see §2.4.1; additionally G3P, glyceraldehyde 3-phosphate; AcCoA, acetyl coenzyme A; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; FPP farnesyl diphosphate; TMTT, (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

2.4.3 Inducibility

To function as an indirect defense mechanism, volatile emissions likely have to be inducible. In analogy to Aesop's fable 'The boy who cried wolf', plants that would call out for help even when not attacked by herbivores would provide little useful information for predators or parasitoids. Indeed, not only the emissions are inducible by wounding and herbivory, this increase in volatile emissions also coincides with elevated expression of genes involved in volatile biosynthesis, including TPS genes, indicating that the increased emissions are likely due to increased synthesis (Van Poecke et al., 2001; Fäldt et al., 2003). It would be interesting to see whether glycoside forms of terpenoids are mobilized upon herbivory.

2.5 Other Defense Mechanisms

2.5.1 Proteinase Inhibitors

As their name suggests, proteinase inhibitors are proteins that interfere with the activity of proteinases. This inhibitory activity may be important for both primary processes such as seed dormancy and protein reserve mobilization, but they have been mainly studied as defense mechanisms. In defenses, they function by inhibiting protease activities of pathogens an herbivores, thereby not only decreasing nutrient uptake but also overstimulating production of proteolytic enzymes in the insect gut which could result in depletion of essential amino acids, both with a negative impact on herbivore performance (Ryan, 1990; Lawrence and Koundal, 2002). Two proteinase inhibitor gene-families have been described in Arabidopsis, one encoding trypsin inhibitors (Clauss and Mitchell-Olds, 2004) and one encoding cysteine proteinase inhibitors (cystatins; Martinez et al., 2005). Of the six trypsin inhibitors detected in the Arabidopsis genome, five are transcribed. Expression of these five genes can be induced *by P. xylostella* feeding. Trypsin inhibitor activity was negatively correlated with *P. xylostella* activity and larval mass but not with leaf damage by *P. xylostella* larvae in a study comparing Arabidopsis genotypes varying in, amongst others, trypsin inhibitor activity (Cipollini et al., 2004). For the cystatin gene family, no function in defense against insects has been reported for Arabidopsis, although transferring a cystatin gene from Arabidopsis into white poplar conferred resistance to the crysomelid beetle *Crysomela populi* (Delledonne et al., 2001).

2.5.2 Polyphenol Oxidase

Other Arabidopsis defenses against insects may include polyphenol oxidase activy (PPOs). Polyphenols act by oxidizing phenolic compounds to reactive quinones, that alkylate essential amino acids and thereby reduce nutritional value. As far as I am aware, the role of PPOs in plant defense against insects has only been demonstrated in vitro (Constabel and Ryan, 1998). In Arabidopsis, PPOs were negatively correlated with *P. xylostella* activity and larval mass and with leaf damage by *P. xylostella* larvae in the study by Cipollini et al.(2004).

2.6 Global Analyses of Defense

In team sports, it is important to know the function of the individual players. However, by focusing the camera on only one player, it is hard to understand the game. For that, you have to look at the whole teams. So far, this review has focused on the different players in the game called Arabidopsis-insect interactions: trichomes, glucosinolates etcetera. However, to get a better understanding on how different players interact, it would be very helpful if we could look at several players at the same time. It would be even better if we could look at the whole team, and possibly find new players. There are several ways of looking at such a broad level, including metabolomics, proteomics and gene-expression profiling.

2.6.1 Transcriptome Analyses

Reymond et al., (2000) were the first to use gene-expression profiling to study Arabidopsis-insect interactions. Together with a follow-up study (Reymond et al., 2004), it showed that caterpillar feeding induces several functional classes of genes, including defense proteins such as a few putative lectins and a cysteine proteinase; phenylpropanoid pathway genes that may be involved in phytoalexin production, radical scavengers or cell wall fortification; oxidative and abiotic stress related genes; and genes involved in relocation of resources. For none of these it is clear how and whether they function in plant defense against insects, or, in the case of the defense proteins, whether they do so in Arabidopsis. Genes with known functions in Arabidopsis defenses, such as those involved in glucosinolate biosynthesis and degradation and genes involved in signaling (see §4.2), are also up-regulated by caterpillars. Only very few genes were down-regulated by caterpillar feeding, and the function of those are unclear (Reymond et al., 2004). Thus, gene-expression studies do not only confirm the role of already known player but are also helpful to indicate new players. Additional information can be obtained by, for example, studying the effect of different herbivores on gene-expression profiles. Reymond et al. (2000) demonstrated that plants infested by two specialist caterpillars from the same genus (*P. rapae* and *P. brassicae*) showed highly similar gene-expression profiles.

They then tested the hypothesis that specialist and generalist herbivores induce different sets of genes, by comparing expression profiles of Arabidopsis plants infested with the specialist herbivore *P. rapae* and the generalist herbivore *Spodoptera littoralis* (Reymond et al., 2004). This because specialists may have found ways to attenuate inducible plant defenses. Interestingly, they found hardly any differences in induced gene-expression, indicating the specialist found ways to deal with plant defenses rather

than to attenuate plant defenses. Indeed, as mentioned before, *P. rapae* can divert glucosinolate degradation from harmfull isothiocyanates to less harmful nitriles (Wittstock et al., 2004), something that *S. littoralis* likely cannot.

In a similar approach, Moran et al. (2002) studied the response of Arabidopsis to aphid feeding. Some of the genes found to be induced by caterpillars in the studies mentioned above were also induced by aphids. These included oxidative stress related genes; a pathogenesis related protein gene (*BGL2*/*PR2*); and signaling related genes. Others, such as the pathogenesis related protein *PR1*, were induced by aphids but not by caterpillars (Moran et al., 2002; Reymond et al., 2004). Aphid feeding also reduced the expression of some genes, including oxidative stress related genes (differential regulation of oxidative stress related genes has been reported before [Kliebenstein et al., 1998]), phenylpropanoid pathway genes and genes involved in signaling (Moran et al., 2002). Again, the function of these aphid-responsive genes in plant-insect interactions is unclear and these studies point out some candidates for further investigation.

Based on the studies above it is clear that inducible Arabidopsis responses to herbivory differ depending on the herbivore species, at least if they differ in feeding mode. However, data collected by different research groups is often hard to compare due to differences in e.g. plant growth conditions. A study by (De Vos et al., 2005) compared gene-expression profiles from plants infested with the caterpillar *P. rapae,* the thrips *Frankliniella occidentalis* or the aphid *M. perzicae* (this experiment also included plants infected with the pathogens *Pseudomonas syringae* or *Alternaria brassicicola*). One of the main conclusions from this study is that the responses to different herbivorous attackers are very dissimilar, with caterpillar and thrips induced changes showing the highest degree of overlap, followed by thrips and aphid induced changes, while caterpillar and aphid induced changes hardly overlapped at all. This corresponds well with different types of damage inflicted by these herbivore species: tissue-feeding caterpillars and cell-feeding thrips cause more mechanical damage than phloem-feeding aphids, which may explain the bigger overlap between caterpillars and thrips compared to caterpillars and aphids. On the other hand, both thrips and aphids puncture individual cells whereas caterpillars disrupt tissue more drastically, which may explain the higher overlap between aphids and thrips compared to aphids and caterpillars. Such a correlation between feeding damage and plant responses has been shown before in a study by Van Poecke et al. (2003), where a parasitoid specialized on *P. rapae* caterpillars was attracted most to volatiles emitted by caterpillar-damaged plants, intermediately to cell-feeding spider mite-infested plants and not at all to aphid infested plants.

2.6.2 Metabolome Analyses

Besides gene-expression profiling, also a metabolomics approach has been used to study Arabidopsis-insect interactions (Van Poecke et al., 2001). This study looked at all metabolites that could be detected in the headspace from caterpillar infested, mechanically damaged or undamaged Arabidopsis plants. Besides terpenoids and glucosinolate-degradation products, several other compounds were detected in the volatile blend emitted by *P. rapae*-infested Arabidopsis. These include fatty acid derived green leaf volatiles such as (*Z*)-3-hexenol, which are negatively associated with aphid growth (Hildebrand et al., 1993) and can function as herbivore repellents or attractants (Reddy and Guerrero, 2000; De Moraes et al., 2001), parasitoid attractants (Whitman and Eller, 1990) and in plant defense signaling (Bate and Rothstein, 1998); and other alcohols, aldehydes and ketones, one of which (penten-3-one, a.k.a. ethyl vinyl ketone) has known signaling properties in Arabidopsis (Alméras et al., 2003). However, none of these functions has been tested in Arabidopsis-insect interactions, with the exception of parasitoid attraction. *C. rubecula* wasps were more attracted to mechanically damaged than to undamaged Arabidopsis. A major difference in the volatile blends of these two odor sources is the abundance of green leaf volatiles in damaged plants, suggesting a role of green leaf volatiles in parasitoid attraction. However, *P. rapae* infested plants emitted less green leaf volatiles than mechanically damaged plants and were preferred by the wasps, indicating that other compounds, such as nitriles, terpenoids and methyl salicylate, play an additional role in parasitoid attraction. The latter also illustrates the difficulty of determining the role of individual compounds in complex volatile blends (Van Poecke et al., 2001).

2.7 Transgenic Plants With Altered Defenses

Several studies on plant-insect interactions have used transgenic plants which were altered in certain defense properties. With respect to Arabidopsis-insect interactions, these studies can be divided into two groups: those that cloned Arabidopsis defense genes into other plant species, and those that cloned defense genes from other plant species into Arabidopsis. As far as I am aware, the only example of Arabidopsis genes introduced into other plant species is the Arabidopsis cystatin gene that was introduced in poplar, mentioned in §2.5.1 (Delledonne et al., 2001). This section will briefly summarize results of foreign genes introduced into Arabidopsis.

2.7.1 Introduction of Proteinase Inhibitors

Besides Arabidopsis proteinase inhibitors being introduced into other species, several publications report foreign proteinase inhibitors introduced into Arabidopsis. De Leo et al., (1998; 2001) introduced the mustard trypsin inhibitor gene *MTI-2* and studied its effect on caterpillar performance, which resulted in weight reduction and increased mortality of *S. littoralis*, increased mortality of *Mamestra brassicae* and 100% mortality of *P. xylostella*. Introduction of a modified cystatin from rice resulted in

increased mortality of the slug *Deroceras reticulatum* (Walker et al., 1999).

2.7.2 Alteration of Terpenoid Profile

The function of terpenoids in direct and indirect defense has been demonstrated by using transgenic Arabidopsis plants expressing a strawberry linalool/nerolidol synthase that can produce both the monoterpene linalool from GDP and the sesquiterpene nerolidol from FDP. When the proteins were targeted to the plastids, this resulted in Arabidopsis plants overproducing mainly the monoterpene linalool and a little bit of the sesquiterpene nerolidol. This indicates that the mevalonate and MEP pathway may not be strictly separated. These plants were less attractive to *M. perzicae* than wild-type plant (Aharoni et al., 2003). When the proteins were targeted to the mitochondria (where FDP is used to generate ubiquinones involved in respiration), this resulted in Arabidopsis plants overproducing the sesquiterpene nerolidol, and to a lesser extend and only in some plants dimethylnonatriene (DMNT). The latter component is a known attractant of *Phytoseiulus persimilis* mites that prey on the herbivorous mite *Tetranychus urticae* (Dicke et al., 1990). Plants overproducing nerolidol or both nerolidol and DMNT were more attractive to *P. persimilis* than wild-type plants, demonstrating that nerolidol can function as a carnivore attractant as well (Kappers et al., 2005).

2.7.3 Alteration of Glucosinolate Profile

Mikkelsen and Halkier (2003) changed the glucosinolate profile of Arabidopsis by introducing *CYP79D2* from cassava that catalyzes the formation of aldoximes from valine and isoleucine, the first dedicated step towards glucosinolate synthesis. *CYP79D2* transgenic Arabidopsis produced valine- and isoleucine-derived glucosinolates not normally found in Arabidopsis. How this affects Arabidopsis-insect interactions was not studied.

2.7.4 Production of a Novel Phytoalexin

Cyanogenic glucosides are related to glucosinolates in that they are also amino-acid derived cyanogenic compounds that are activated upon tissue-disruption by enzymatic removal of glucose. Cyanogenic glucosides are normally not produced by cruciferous plants. Introduction of the three *Sorghum bicolor* genes required for synthesis of the cyanogenic glycoside dhurrin from tyrosine resulted in dhurrin synthesis in Arabidopsis and conferred resistance to larvae from the flea beetle *Phyllotreta nemorum* (Tattersall et al., 2001).

2.7.5 Production of a Novel Toxin

Photorhapdus luminescens is a symbiont bacterium of entomopathogenic nematodes (nematodes that infect insects). This bacterium produces several protein toxins that are toxic to caterpillars, including toxin A. Arabidopsis plants producing toxin A by insertion of a modified version of the *tcdA* gene from *P. luminescens* showed close to 100% mortality of *M. sexta* larvae, compared to about 15% on wild-type plants (Liu et al., 2003).

2.7.6 Induction of Anthocyanins

The last example of transgenic plants with enhanced resistance against insect herbivores comes from Arabidopsis plants that show constitutive overexpression of the Arabidopsis PAP1 transcription factor. This transcription factor regulates the biosynthesis of phenylpropanoids including anthocyanins. PAP1 overexpressing plants showed slightly reduced feeding rates of generalist *Spodoptera frugiperda* but not of *T. ni* and no effect on larval weight for either lepidopteran species. Transgenic plants showed reduced fecundity in absence of herbivory, indicating a cost of constitutively enhanced defenses. However, the exact cause of increased resistance and reduced fecundity were unclear (Johnson and Dowd, 2004).

3. INDUCTION OF DEFENSES

As mentioned previously, many defense mechanisms are not only constitutively present but can also be enhanced upon recognition of attack. Such inducibility may not only serve to reduce costs of defense, but also may help to prevent the buildup of resistance against these defenses in herbivores (Agrawal and Karban, 1999). This section focuses on elicitation and signal transduction of inducible defenses.

3.1 Elicitation

All herbivorous insects wound the plant they are feeding on, although there are large differences in the extent of wounding caused by e.g. tissue-feeding caterpillars, cell-feeding thrips and phloem-feeding aphids. Indeed, mechanical wounding is able to induce many responses that are also induced by herbivory (e.g. Mithofer et al., 2005). Besides wounding, other factors can indicate the presence of herbivorous insects. For example, fatty-acid amino-acid conjugates (FACs) and enzymes such as ß-glucosidase and glucose oxidase may be present in the regurgitant of caterpillars and can influence plant responses (with either positive or negative effects from the plant's point of view) (Mattiacci et al., 1995; Alborn et al., 1997; Halitschke et al., 2001; Musser et al., 2002). Similarly, aphid and thrips saliva is known to contain many enzymes that are thought to influence plant defense (Cherqui and

Tjallingii, 2000; Kindt et al., 2003). Thus, both wounding and biochemical elicitors are likely involved in triggering plant defenses.

3.1.1 Wounding-Induced Responses

Wounding produces many different signals. First of all, damaged cells release their contents; secondly the damaged cell-wall releases signaling compounds such as oligosaccharides; and thirdly undamaged cells near the wound site will experience stresses such as pressure differences (reviewed by De Bruxelles and Roberts, 2001; León et al., 2001). The involvement of cell-wall components in defense resistance is illustrated by the enhanced resistance against aphid *M. persicae* in the Arabidopsis *cev1* mutant. CEV1 is a cellulose synthase and mutation of *CEV1* affects cell-wall formation. Enhanced resistance is possibly caused by a higher concentration of cell-wall derived elicitors triggering plant defenses (Ellis et al., 2002a/b).

The initial elicitors generated upon wounding activate an extensive signaling network triggering a diversity of responses. For example, in Arabidopsis wounding induces 1) signaling compounds such as the plant stress hormones jasmonic acid and ethylene; 2) the expression of a large number of genes; 3) biochemical and structural changes, including trichome density and volatile emissions, and 4) attraction of insects, such as parasitoid wasps (Rojo et al., 1999; Reymond et al., 2000; Van Poecke et al., 2001; Fäldt et al., 2003; Traw and Bergelson, 2003; Delessert et al., 2004; Devoto et al., 2005). Similarly, caterpillar-feeding induces 1) signaling compound such as jasmonates and ethylene; 2) gene-expression of a large number of genes; 3) biochemical and structural changes, including glucosinolate biosynthesis, trypsin inhibitor biosynthesis and volatile emissions; and 4) attraction of insects, such as parasitoid wasps (Van Poecke et al., 2001; Stotz et al., 2002; Clauss and Mitchell-Olds, 2004; Reymond et al., 2004; De Vos et al., 2005; Mewis et al., 2005). As previously mentioned, differences in mechanical wounding inflicted by herbivores with different feeding strategies are correlated with differences in induction of plant hormone levels, gene-expression, and parasitoid attraction (Van Poecke et al., 2001; De Vos et al., 2005). These parallels strongly suggest that wounding can account for many of the responses induced by herbivory. The responses mentioned above also show some differences between wounding and herbivory, which can be partially explained by lack of knowledge. For example, it seems likely that glucosinolate and trypsin inhibitor biosynthesis can also be induced by wounding and trichome density can probably be induced by herbivory but this has not been reported yet. However, some effects that have been studied in parallel for both treatments indicated differences between wound- and herbivory-induced responses of Arabidopsis. For example, Reymond et al. (2000) found that wounding induced the expression of many water stress-related genes, whereas caterpillar feeding did not. Conversely, caterpillar feeding induced the expression of several genes that were not induced by wounding, including defense hormone (jasmonates, see below) and glucosinolate biosynthesis genes, (Reymond et al., 2004). Additionally, caterpillar feeding resulted in the emission of volatile compounds such as methyl salicylate and terpenoids that were not induced by wounding (Van Poecke et al., 2001). These findings suggest that herbivore-derived elicitors are involved in elicitation of plant responses. However, it should be noted that the mechanical damage inflicted by herbivores is hard to mimic (Baldwin, 1990). First of all, the devices commonly used for wounding are much more blunt than the mandibles of a caterpillar and secondly, mechanical damage is often inflicted in a very different spatial and/or temporal pattern compared to herbivory, for example by damaging the plant only at one time point. Herbivore damage on the other hand gradually increases over time. Illustrating the difficulty of mimicking herbivory by mechanical damage, some of the genes reported by Reymond et al. (2004) induced by caterpillar feeding but not by wounding are reported to be induced by wounding in other studies (Reymond and Farmer, 1998; Reymond et al., 2000; Delessert et al., 2004), most likely reflecting differences in mechanical damage.

3.1.2 Herbivore-Derived Elicitors

To get a better understanding of which responses to herbivory are elicited by wounding and which are caused by other elicitors, an often used method is to apply herbivore-derived elicitors, for example in the form of regurgitant, to mechanically damaged plants. In Arabidopsis, application of caterpillar-derived regurgitant resulted in induced gene-expression (Berger et al., 2002; Reymond et al., 2004), parasitoid attraction (Van Poecke and Dicke, 2003) and jasmonate biosynthesis (Van Poecke, unpublished results). However, it is unclear to what extent compounds in the regurgitant come into contact with plant tissue during herbivory and to what extent different plant species are responsive to these compounds.

3.1.2.1 Fatty Acid Amino Acid Conjugates. Many studies strongly indicate a role for fatty acid amino acid conjugates (FACs) in eliciting herbivory-related responses. For example, differences in induced gene-expression in *Nicotiana attenuata* to generalist and specialist herbivores are reflected in differences in FAC composition of the regurgitant of these herbivores and application of these different regurgitants to *N. attenuata* mimicked the effect of herbivory by the different herbivore species (Voelckel and Baldwin, 2004). However, not all plant species are responsive to FACs. In lima bean, which is not responsive to FACs (Spiteller et al., 2001), careful mimicking of only the mechanical part of herbivore damage resulted in the emission of a volatile blend highly similar to that of herbivore infested plants (Mithofer et al., 2005). There are several indications that Arabidopsis is not responsive to FACs: 1) specialist and generalist herbivores elicit very similar responses in Arabidopsis (Van Poecke et al., 2003;

Reymond et al., 2004), which can either be explained by a very similar FAC content or by Arabidopsis being non-responsive to FACs; 2) FAC biosynthesis requires plant derived linolenic-acid, therefore regurgitant from caterpillars feeding on Arabidopsis plants lacking linolenic acid should lack FACs, however this regurgitant was just as effective in inducing gene-expression in wild-type plants as 'normal' regurgitant (Reymond et al., 2004).

3.1.2.2 Glucose Oxidase and ß-Glucosidase. Other elicitors include glucose oxidase from *Helicoverpa* species, which oxidizes D-glucose to D-gluconic acid and hydrogen peroxide and ß-glucosidase from *P. brassicae*, which removes glucose from unknown substrates (Mattiacci et al., 1995; Musser et al., 2002). With respect to glucose oxidase, it is unknown whether regurgitant from the caterpillar species used in the Arabidopsis studies (*P. rapae, P. brassicae, P. xylostella* and *S. littoralis*) contain glucose oxidase, but regurgitant from another member of the *Spodoptera* family (*S. litura*) does not (Zong and Wang, 2004). There are no published effects studying ß-glucosidase on Arabidopsis, although treating mechanically damaged Arabidopsis with ß-glucosidase did not result in any changes in volatile emissions compared to mechanical damage alone (Van Poecke, unpublished results).

In short, there are indications that elicitors may play a role in caterpillar-induced changes in Arabidopsis, but this is far from being certain. Perhaps the most convincing data on elicitor involvement in Arabidopsis-herbivore interactions comes from a study on aphid-induced changes: aphids induced a dramatic change in the gene-expression profile of Arabidopsis, much more so than either thrips or caterpillars, whereas caterpillar or thrips feeding results in much more mechanical damage (De Vos et al., 2005).

Some elicitors that play a role in herbivory-induced responses may not directly originate from the herbivore itself, but rather from microorganisms associated with the herbivore. Not only do insects act as vectors of plant diseases, they also carry symbionts and commensals in their digestive system, which may influence plant-insect interactions (Sobek and Munkvold, 1999; Spiteller et al., 2000; Mitchell, 2004; Belliure et al., 2005; but see Lait et al., 2003). Transmission of plant diseases by herbivorous insects may go unnoticed as many pathogens do not result in a visible phenotype (Dardick et al., 2000), but may influence plant-insect interactions.

3.2 Signal Transduction

Upon elicitation, information needs to be processed through a signaling network in order to trigger responses. Most research on signal transduction in plant defense against insects centered around three classes of hormones: jasmonates (a.k.a oxylipins), salicylates and ethylene, but there are indications that other hormones are involved. Several lines of evidence can indicate the involvement of a hormone in plant defense against herbivores: 1) the hormone is induced by herbivory; 2) application of the hormone to the plant affect resistance against herbivores; and 3) mutation of the hormonal pathway affects resistance against herbivores. In this section I will introduce the hormone classes and discuss the roles of the hormone class in plant following the above mentioned lines of evidence. Note that other chapters in *TAB* cover the roles of these hormones in both plant-microbe interactions and normal development:

"Abscisic Acid Biosynthesis and Response"
Ruth R. Finkelstein and Christopher D. Rock

"Ethylene"
G. Eric Schaller and Joseph J. Kieber

"The Arabidopsis Thaliana-Pseudomonas Syringae Interaction"
Fumiaki Katagiri, Roger Thilmony, and Sheng Yang He

"Interactions between Xanthomonas Species and Arabidopsis thaliana"
C. Robin Buell

"The Oxylipin Pathway in Arabidopsis"
Robert A. Creelman and Rao Mulpuri

I will start with the most important hormonal class in plant-insect interactions: jasmonates.

3.2.1 Jasmonates

3.2.1.1 Biosynthesis. Jasmonates are a group of oxidized, fatty-acid derived compounds with hormonal functions, the most famous member being jasmonic acid (JA; figure 6). The biosynthetic pathways of jasmonates start with the liberation of linolenic acid (18:3 fatty acid) or 16:3 fatty acid from membrane lipids by phospholipases. A possible explanation of the reduced emission of volatiles by lox2-silenced plants is the lack of trichomes of these plants. This lack of trichomes is not due to JA deficiency but because these mutants were created in a hairless Columbia background, although this was not mentioned in the original publication (Bell et al., 1995). This indicates that trichomes may play a major role in the emission of volatiles by Arabidopsis. Several phospholipases have been associated with JA biosynthesis: *DAD1, PLA-IIA,* and *PLD-alpha1* all encode wound-inducible phospholipases (Wang et al., 2000; Ishiguro et al., 2001; Rietz et al., 2004). However, only mutation of *PLD-alpha1* has been associated with reduced levels of wound-induced JA (Wang et al., 2000). The 18:3 and 16:3 fatty acids are subsequently used by lipoxygenases (LOX), allene-oxide synthase (AOS) and allene-oxide cylcase (AOC) to produce 12-oxophytodienoic acid (OPDA) and dinor-oxophytodienoic acid (dnOPDA) (from 18:3 and 16:3 respectively). OPDA and likely also dnOPDA can be metabolized to JA through the action of 12-oxophytodienoic acid reductase (OPR) and a

ß-oxidase complex of limited specificity. JA can be modified in several ways, e.g. by JA methyl transferase (JMT), resulting in methyl-jasmonate (MeJA) and by JAR1, resulting in amino-acid conjugated JA, especially with isoleucine (JA-Ile). A side-branch of the oxylipin pathway uses the products of LOX to produce the green leaf volatiles, through the action of hydroperoxide lyase (HPL) and alcohol dehydrogenase (ADH). OPDA, JA, MeJA, JA-Ile and green leaf volatiles all have demonstrated, often distinct functions in plant defense (for reviews on oxylipin biosynthesis and function see Creelman and Mulpuri, 2002; Schaller et al., 2004; Van Poecke and Dicke, 2004; also see R. Liechti and E.E. Farmer's Science STKE connection map at http://stke.sciencemag.org for an interactive resource).

3.2.1.2 Induction. In Arabidopsis, jasmonate biosynthesis is stimulated by wounding, caterpillar and thrips feeding (Stotz et al., 2002; Reymond et al., 2004; De Vos et al., 2005). Correspondingly, expression of jasmonate biosynthesis genes can be induced by wounding (demonstrated for LOX2, AOS, AOC and HPL), and caterpillar feeding (demonstrated for LOX2, AOS, HPL and OPR3) (Reymond et al., 2000; Stotz et al., 2000; Stenzel et al., 2003; Reymond et al., 2004). Aphid feeding also induced the expression of LOX2, however, no induction of JA-levels could be detected (Moran and Thompson, 2001; Moran et al., 2002; De Vos et al., 2005). Besides JA biosynthesis genes, also many JA-responsive genes are induced by caterpillar and thrips-feeding. For *P. rapae*, 48-55% of the insect responsive genes are also MeJA responsive and for thrips this was even higher: 69% (Reymond et al., 2004; De Vos et al., 2005). Interestingly, only about half of the caterpillar-induced JA responsive genes were also induced by thrips, indicating differences in responses to caterpillar and thrips-feeding downstream of JA (De Vos et al., 2005).

3.2.1.3 Application. Application of jasmonates affects trichome formation, glucosinolate biosynthesis and terpenoid emissions. For example, Traw and Bergelson (2003) demonstrated for several Arabidopsis accessions that spraying with JA induced trichome formation in new leaves. Similarly, several studies have shown induction of glucosinolates by MeJA treatment, with varying effects between different glucosinolate classes and Arabidopsis accessions. Interestingly, the pattern of MeJA-induced glucosinolates (mainly indole glucosinolates) does not correspond with the pattern induced by the caterpillar *S. exigua,* or the aphids *M. persicae* and *B. brassicae* (mainly aliphatic glucosinolates) (Brader et al., 2001; Kliebenstein et al., 2002; Mikkelsen et al., 2003; Cipollini et al., 2004; Mewis et al., 2005). Spraying with JA induced the emission of several volatile compounds, with the most dramatic increases in terpenoid emissions (Van Poecke, 2002; Fäldt et al., 2003). Additionally, OPDA, JA or MeJA treatment induced many genes that are also induced by wounding and/or caterpillar feeding, including glucosinolate and terpenoid biosynthesis genes and defense protein genes, although there are also differences between the effects of these treatments (Fäldt et al., 2003; Reymond et al., 2004). It is therefore not surprising that application of jasmonate affects Arabidopsis-insect interactions: JA-treatment reduces larval mass of *S. exigua* and induces the attraction of *C. rubecula* parasitoids (Van Poecke and Dicke, 2002; Cipollini et al., 2004).

3.2.1.4 Mutants. Many studies on jasmonates and Arabidopsis-insect interactions have used mutant plants. In Arabidopsis, several mutants are either affected in oxylipin biosynthesis or downstream responses. Examples of biosynthesis mutants are the *fad3-2fad7-2fad8* triple mutants that cannot make JA or its precursors OPDA and dnOPDA because the biosynthetic pathways leading to 16:3 and 18:3 unsaturated fatty acids have been blocked (McConn and Browse, 1996); *lox2* cosuppressed plants that do not show increased JA levels upon wounding (Bell et al., 1995); *opr3* mutants that cannot convert OPDA or dnOPDA into JA (Stintzi et al., 2001) and *jar1* mutants that are unable to conjugate JA to isoleucine, which is necessary for some but not all JA responses (Staswick and Tiryaki, 2004).The downstream mutants include *coi1* mutants that are impaired in all known JA responses (Devoto et al., 2002); and *mpk4* mutants that do not show JA-induced inhibition of the SA pathway (Petersen et al., 2000). A more detailed discussion of the effects of these mutations on Arabidopsis-insect interactions follows next (and see figure 7).

3.2.1.4.1. *fad3-2fad7-2fad8* - affecting overall oxylipin levels. By using a *fad3-2fad7-2fad8* triple mutant, McConn et al. (1997) demonstrated that jasmonates are involved in defense against insects in Arabidopsis. The triple mutant is more susceptible to root-feeding larvae from the common fungus gnat *B. impatiens*, but resistance could be restored to nearly wild-type levels by applying MeJA. On the other hand, Cipollini et al. (2004), could not detect differences in larval growth rate and fresh weight of *S. exigua* after 48 h of feeding on wild-type plants or *fad3-2fad7-2fad8* triple mutants.

3.2.1.4.2. *LOX2* silenced plants - affecting inducible jasmonate levels. Whereas, *fad*-triple mutants affect all 16:3 and 18:3 derived oxylipin products, including jasmonates and green leaf volatiles, *lox2* silenced plants only affect wound-induced jasmonate levels (Bell et al., 1995). Using these plants, Van Poecke et al. (2002) reported reduced attraction of parasitoid wasps after herbivory. However, analyses of volatile emissions showed reduced emissions of all volatiles detected, including constitutively present, non-inducible compounds that are not likely to depend on induced JA levels, suggesting that the reduced attraction of parasitoid wasps cannot be explained by a loss of JA inducibility (Van Poecke, 2002). A possible explanation could be the absence of trichomes on lox2 silenced plants. Although not mentioned in the original publication (Bell et al., 1995), lox2 silenced plants were generated in a hairless Columbia background. The effect on parasitoid attractions therefore appears to be due to lack of trichomes rather

than lack of inducible JA. Analyses of oxylipin levels in these plants showed that lox2 cosuppressed plants still show increased accumulation of jasmonates after *P. rapae* feeding, although somewhat less then wild-type plants, indicating that silencing may not be complete (Van Poecke, unpublished results).

3.2.1.4.3. *opr3* - affecting JA levels. Further dissection of the role of oxylipins in defense against insects has been

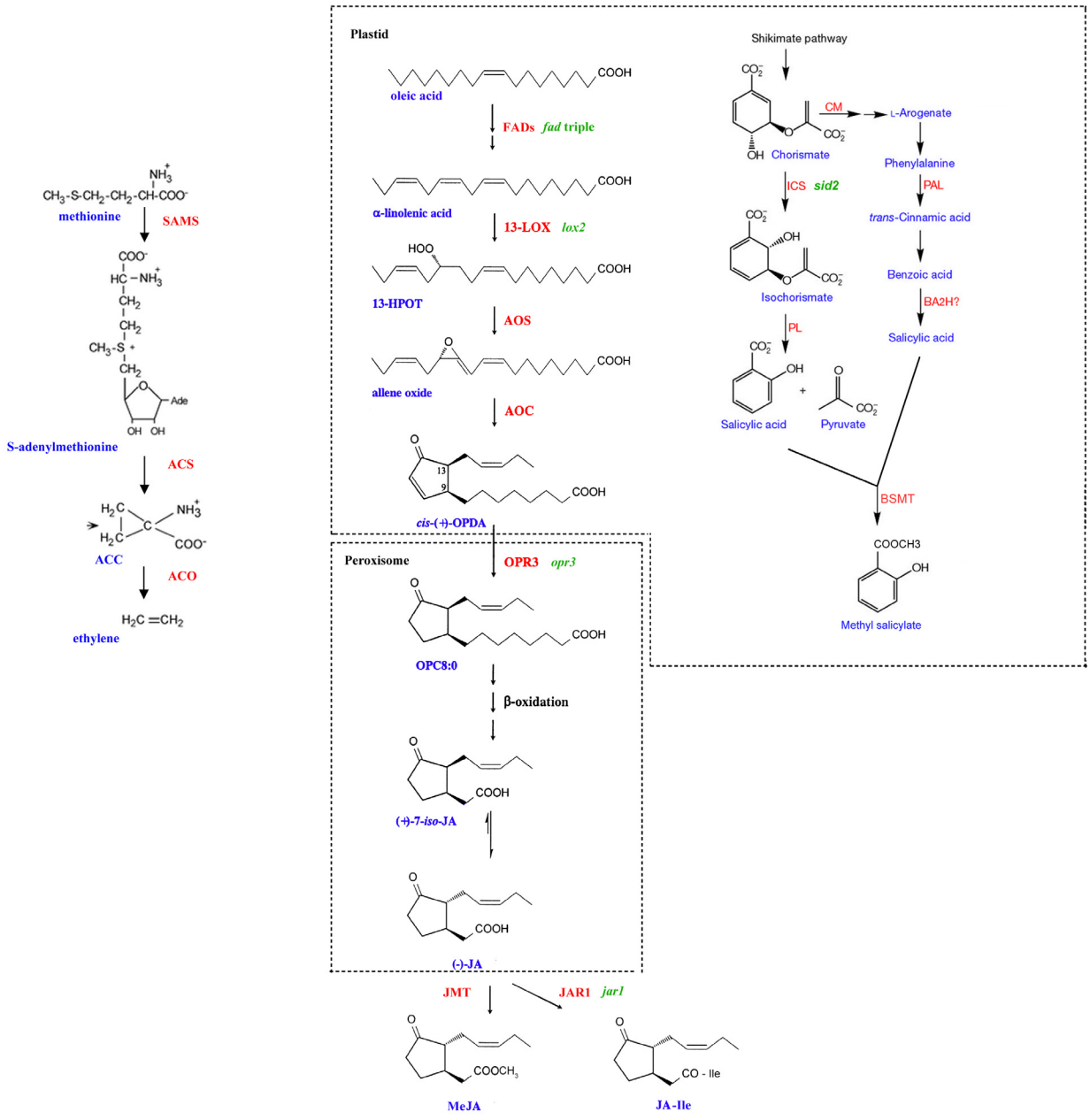

Figure 6. Biosynthesis of ET, JA and SA. From left to right: biosynthesis of ET, jasmonates and SA. Enzymes are indicated in red, mutants in green and compound names in blue. For explanation of abbreviations see §3.2.2.1 (ET), §3.2.1 (jasmonates) and §3.2.3.1 (SA); additionally FADs, fatty acid desaturases; 13-HPOT, 13(S)-hydroperoxy linolenic acid; OPC8:0, 3-oxo-2(2_(Z)-pentenyl)-cyclopentane-1-octanoic acid; BA2H, benzoic acid-2 hydroxylase. Figure adapted with permission from Schaller and Kieber (2002); Schaller et al. (2005); and Wildermuth et al. (2001).

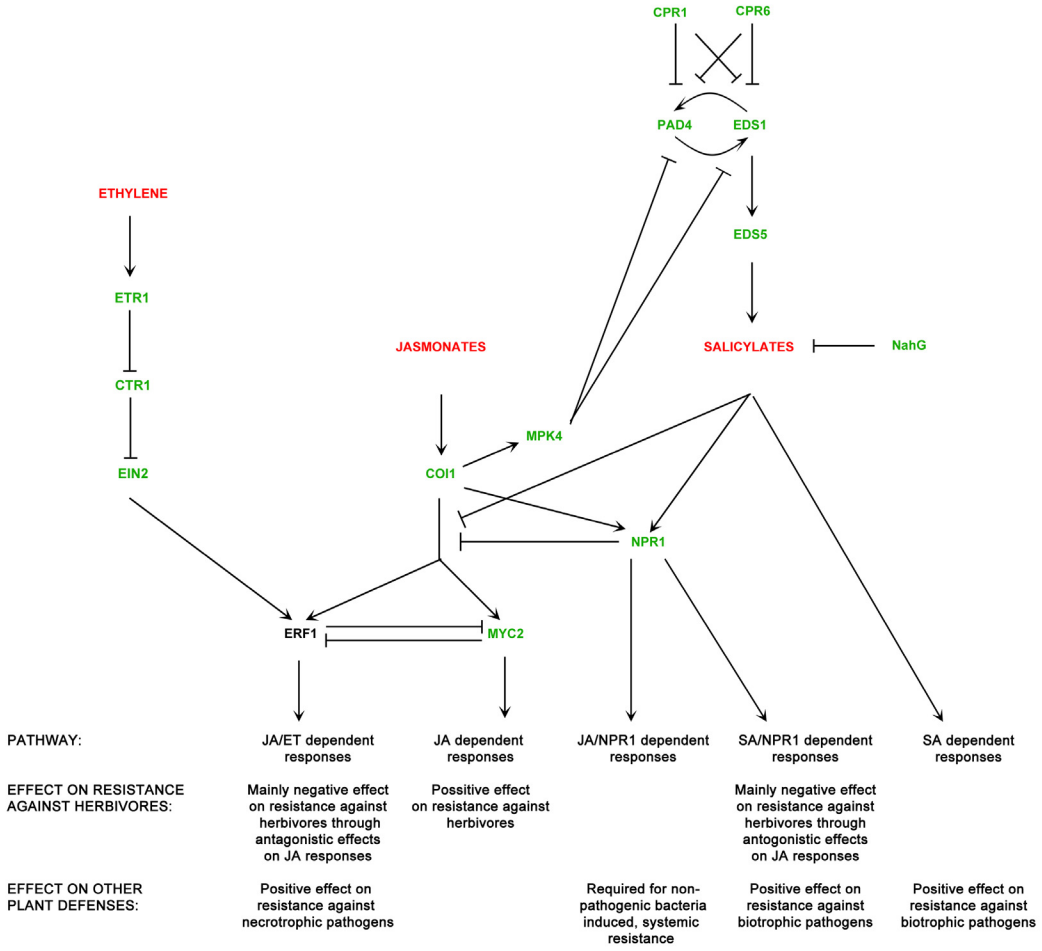

Figure 7. Signaling networks. A simplified scheme of ET, JA and SA signaling and their interactions. Hormones are depicted in red, proteins for which mutants were discussed in section 3.2 are depicted in green. Figure partially adapted from de Vos (2006).

performed by using *opr3* mutant plants. Stinzi et al. (2001) used *opr3* mutants, which are in a Ws-0 background, to demonstrate that biosynthesis of JA from OPDA is not required for defense against *B. impatiens* and that JA-precursors such as OPDA are sufficient to retain resistance.

3.2.1.4.4. *jar1* - affecting JA conjugation to amino acids. So far, there is no indication that amino-acid conjugation of JA is involved in Arabidopsis-insect interactions. Comparing wild-type and *jar1-1* plants, no difference could be detected in constitutive or JA-induced glucosinolate levels (Cipollini et al., 2004) or JA-induced trichome formation (Traw and Bergelson, 2003). The *jar1* mutation also did not affect larval growth rate and fresh weight of *S. exigua* larvae after 48 h of feeding (Cipollini et al., 2004), M. perzicae population growth during 6 consecutive days (Dong et al., 2004), or parasitoid attraction to *P. rapae*-infested plants (Van Poecke and Dicke, 2003).

3.2.1.4.5. *coi1* - affecting signal-transduction downstream of jasmonates. COI1 is an F-box protein involved in targeting proteins for degradation. It is hypothesized that the targets of COI1 include repressors of jasmonate responses that act downstream of jasmonate biosynthesis (Devoto et al., 2002). Loss of COI1 function, such as in the *coi1-1* mutant, affects all known responses downstream of jasmonates, with the possible exception of responses depending on the electrophilic properties of some oxylipins (Alméras et al., 2003). Both constitutive and MeJA induced levels of indole and some aliphatic glucosinolate levels, as well as MeJA induced expression of glucosinolate biosynthesis genes, are reduced or abolished in *coi1* (Brader et al., 2001; Mikkelsen et al., 2003; Mewis et al., 2005). Of the genes induced by *P. rapae* feeding, 67-84% was COI1 dependent (Reymond et al., 2004). The *coi1* mutants show increased growth rates of *S. littoralis* and *S. exigua* and are more susceptible to *B. impatiens* and to *M.*

perzicae and *B. brassicae* (Stintzi et al., 2001; Stotz et al., 2002; Mewis et al., 2005).

3.2.1.4.6. *mpk4* - affecting JA/SA interactions. MPK4 is a map-kinase that is also required for several downstream responses of JA. The *mpk4* mutant, which is in a Ler background, shows enhanced levels of SA and SA-inducible responses, likely caused by alleviation of JA repression of SA. Moreover, *mpk4* mutants are impaired in the induction of MeJA responsive genes and this inhibition is independent of SA. Thus, *mpk4* mutants are affected in JA signaling in two ways: by direct inhibition of JA inducible responses and by elevated SA responses which in turn antagonize JA responses (see below). Induction of glucosinolate levels by MeJA is inhibited in these mutants (Mikkelsen et al., 2003). Andreasson et al. (2005) mention unpublished results showing that *mpk4* mutants are more susceptible to herbivore feeding. It is unclear whether these effects are caused by direct JA signaling effects; by enhanced SA inhibition of JA responses; or by both.

In short, oxylipins like OPDA and JA are important for resistance against insect herbivores with different modes of feeding, including caterpillars, thrips and aphids. The role of OPDA and JA seems to be redundant, meaning that OPDA can take over the role of JA and vice versa, although the role of OPDA has so far only been established for resistance against *B. impatiens*. Besides having overlapping functions, both JA and OPDA also have distinct functions. For example, JA is essential for male fertility and OPDA can induce the expression of genes which are JA and COI1 independent, possibly through its electrophilic properties (Feys et al., 1994; McConn and Browse, 1996; Stintzi et al., 2001). Whether the specific properties of JA and OPDA are important for plant defense against insects is unclear. If OPDA can account for oxylipin-dependent resistance against herbivores in general, conjugation of oxylipins to isoleucine through JAR1 is not likely to be important, as OPDA is not a substrate for this enzyme (Staswick and Tiryaki, 2004). Indeed no effects of JAR1 on Arabidopsis insect interaction have been reported. The downstream component COI1 is essential for most oxylipin-dependent responses, either mediated to OPDA or JA. The contradictory results of reduced performance of *S. exigua* on *coi1* but not *fad* triple mutants, may indicate an oxylipin-independent effect of COI1 on herbivory by *S. exigua*, but could also be due to difference in methods. For example, cut-off rosettes were used in the *fad*-triple experiment, whereas the *coi1* mutants were left intact (Cipollini et al., 2004; Mewis et al. 2005). Jasmonates also interact with other hormonal pathways, including the ethylene and salicylate pathways, which are discussed next.

3.2.2 Ethylene

3.2.2.1 Biosynthesis. Ethylene is a plant hormone derived from methionine by the consecutive action of S-adenylmethionine synthase (SAMS), 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO; figure 6). ACC synthase activity is the first dedicated step towards ethylene biosynthesis. In plant defense, ethylene is mainly known for its positive role in resistance against necrotrophic pathogens, in concert with JA (reviewed by Glazebrook, 2005). However, there are several indications that ET also affects plant-herbivore interactions (for a review of ethylene biosynthesis see Schaller and Kieber, 2002; also see A.N. Stepanova and J.M. Alonso's Science STKE connection map at http://stke.sciencemag.org for an interactive resource).

3.2.2.2 Induction. Both wounding and herbivory by caterpillars results in induction of ethylene, whereas thrips feeding has little and aphid feeding no influence on ET levels (Rojo et al., 1999; De Vos et al., 2005). Ethylene biosynthesis genes are induced by wounding (demonstrated for several ACS and ACO1) as well as caterpillar feeding (demonstrated for SAMS2 and ACO1), and the same holds true for ethylene responsive genes (Reymond et al., 2004; Tsuchisaka and Theologis, 2004).

3.2.2.3 Application. Treatment with the ethylene precursor ACC negatively affected the biosynthesis of MeJA-inducible aliphatic and indole glucosinolates and the expression corresponding biosynthetic genes. Conversely, treatment with MeJA negatively affected the biosynthesis of an ET-inducible indole glucosinolate (Mikkelsen et al., 2003). Treatment of Arabidopsis with ethephon, an ethylene releasing compound, resulted in decreased resistance against the generalist *S. littoralis*, possibly due to its negative effect on JA-induced defenses, but not against the specialist *P. xylostella* (Stotz et al., 2000).

3.2.2.4 Mutants. Several mutants have been used to study Arabidopsis-insect interactions. All mutants described here are downstream regulators of ethylene signaling. These include *etr1-1* which is disrupted in ET perception (Wang et al., 2003), *ctr1* which shows constitutive activation of ET responses (Huang et al., 2003), and *ein2-1* and *hsl1-1* which show mutations in positive transducers of ET signaling (Lehman et al., 1996; Alonso et al., 1999) (figure 7).

3.2.2.4.1. *etr1-1* - affecting ethylene perception. ETR1 encodes one of the five ethylene receptors in Arabidopsis. ETR1 interacts with CTR1 and upon binding of ET inhibits CTR1 (Wang et al., 2003). The *etr1-1* mutant shows enhanced induction of indole glucosinolates by generalist and specialist aphids, but not by *S. exigua*. This mutation had a negative impact on caterpillar but not aphid performance (Mewis et al., 2005). Additionally this mutant was used in a study on crosstalk between the bacterial pathogen harpin protein and resistance against *M. persicae*. Harpin induced ETH (and SA and reduces JA) concentration in plants and induces resistance against *M. persicae*. This induced resistance against *M. persicae* is dependent on the ethylene-receptor ETR1 (Dong et al., 2004).

3.2.2.4.2. *ctr1-1* - affecting signal transduction downstream of ethylene. CTR1 is a protein kinase that acts as negative regulator of ethylene responses and possibly targets EIN2. The *ctr1-1* mutant therefore shows constitutive activation of ethylene responses and has a stunted phenotype (Kieber et al., 1993; Alonso et al., 1999; Huang et al., 2003). As expected, the *ctr1-1* mutant shows decreased levels of MeJA inducible glucosinolates, fitting nicely with the antagonistic effect of exogenous applied ACC on MeJA inducible glucosinolates. Additionally, the *ctr1-1* mutant also shows reduced levels of a glucosinolate that is induced by ACC (Mikkelsen et al., 2003).

3.2.2.4.3. *ein2-1* - affecting signal transduction downstream of ethylene. Similar to the *coi1-1* mutant for jasmonate response, the *ein2-1* mutant affects all known ethylene responses and is thus completely insensitive to ethylene. Although *EIN2* is cloned, its aminoacid sequence is rather unique and its function remains unclear (Alonso et al., 1999). The *ein2-1* showed the higher basal indole glucosinolates levels. Additionally, *ein2-1* showed enhanced resistance against the generalist *S. littoralis* but not against the specialist *P. xylostella* (Stotz et al., 2000). In contrast, *ein2-1* did not show harpin-induced resistance against *M. persicae* (Dong et al., 2004).

3.2.2.4.4. *hls1-1* - affecting ethylene-auxin interactions. HLS1 shows similarities to N-acetyltransferases and possibly acetylates proteins involved in auxin responses. HSL1 also acts downstream of ethylene and is thought to function as a regulator of auxin-activity involved in differential cell-growth involved in apical hook formation (Lehman et al., 1996). Interestingly, Stotz et al. (2000) found that the *hls1-1* mutant showed a similar phenotype with respect to caterpillars as the *ein2-1* mutant: enhanced resistance against the generalist *S. littoralis* but not against the specialist *P. xylostella*, suggesting that HLS1 not only affects morpholocial features, but also plant defenses. Whether auxin is involved in this effect of HLS1 is unclear.

3.2.2.5 Interaction Between JA and ET Pathways. Summarizing, it appears that ethylene is involved as a negative regulator of caterpillar-induced defenses, affecting generalist but not specialist caterpillars. That ethylene did not affect the performance of a specialist caterpillar may not be because ethylene does not influence defenses in this interaction but rather that these defenses are not effective against this specialist. This seems plausible considering that the effect of ethylene on herbivore performance is likely mediated at least partly through glucosinolates, which are not effective against *P. xylostella*. Thus, ethylene seems to exert an inhibitory effect on herbivory-induced, JA-dependent responses such as, but not limited to, glucosinolate production. Lorenzo et al. (2004) demonstrated that JA responses can be split into two groups, mediated either by AtERF1 (ethylene responsive factor 1) or AtMYC2 (=JAI1) which act antagonistically. Thus stimulation of AtERF1 by the combination of ET and JA inhibits the JA responses mediated through AtMYC2 and stimulation of AtMYC2 through JA inhibits the responses mediated through AtERF1. Recently, De Vos (2006) showed that caterpillar feeding or regurgitant treatment induces the AtMYC2 pathway and inhibits the AtERF1 pathway, possibly through concerted action of JA and abscisic acid (ABA), as demonstrated using *jin1-2* (defective in AtMYC2 and originally identified as a jasmonate-insensitive mutant) and *aba2-1* (ABA biosynthesis mutant). This fits well with the idea that ET has a negative effect (through AtERF1) on defenses against caterpillars. Although ET is induced by caterpillar feeding, the plant may attenuate the negative effect of ET through induction of AtMYC2. Apparently, this inhibition is not complete, which would explain why ET mutants are more resistant against generalist caterpillars.

With respect to aphids, the effect of ET on plant defenses is less clear. ET is a negative regulator of aphid-induced defenses, but this does not seem to affect aphid performance. Induced resistance against aphids by application of the bacterial harpin elicitor depends on ET. This suggests that ET acts as a positive mediator of resistance against aphids. However, this might be specific to Arabidopsis-pathogen-aphid interactions. The *cev1* mutant, that through changes in cell wall properties (possibly higher concentrations of cell-wall derived elicitors) showed increased levels of oxylipins, especially JA, and ET, also showed enhanced resistance against *M. persicae* (Ellis et al., 2002a). However, it is unclear whether the latter can be mainly attributed to JA, ET, or a combination of both. Thus, there are no clear indications that ET plays a stimulatory part in the normal defense response of Arabidopsis against aphids.

In short, JA/ET responses appear to be mainly important for plant defense against necrotrophic pathogens and acts antagonistically to plant defense against insects. Conversely, the 'JA-only' pathway appears to be mainly important for plant defense against insects and acts antagonistically to plant responses to necrotrophic pathogens (figure 7).

3.2.3 Salicylates

3.2.3.1 Biosynthesis. Salicylates, such as salicylic acid (SA), are especially known from their role in defense against biotrophic pathogens (Glazebrook, 2005). There appear to be two biosynthetic pathways, the phenyl-propanoid pathway and the isochorismate pathway (figure 6). Both use the precursor chorismate as a substrate. In the isochorismate pathway, chorismate is used by isochorismate synthase (ICS1) and pyruvate lyase (PL) to produce SA. In the phenyl-propanoid pathway, chorismate is used by chorismate mutase (CM), which through intermediates L-arogenate, phenylalanine, trans-cinnamic acid, and benzoic acid (BA) results in the production of SA (Wildermuth et al., 2001). SA can subsequently be used by benzoic/salicylic acid methyl transferase (BSMT) to produce the more volatile methyl-salicylate (MeSA) (Chen et al., 2003a). Additionally, both BA and SA can be stored in an inactive glucoside form (Seo et al., 1995; Chong et al., 2001). Studies on the role of salicylates in plant-insect

interactions have mainly focused on their attenuating effect on jasmonate-induced responses. However, there is also evidence for a role of salicylates as a positive mediator of plant defense against insects (De Boer and Dicke, 2004).

3.2.3.2 Induction. Studies on endogenous SA levels after herbivore-attack have produced different results. De Vos et al (2005) found no induction of SA by *P. rapae, F. occidentalis* or *M. persicae* feeding. This corresponds well with the finding by Reymond et al. (2000, 2004) that wounding or feeding *by P. rapae, P. brassicae* or *S. littoralis* did not result in the upregulation of SA-responsive genes. On the other hand, Stotz et al. (2002) mentioned induced SA levels upon *S. littoralis* but not *P. xylostella* feeding in *Ler-0*, although these data have not been published. Van Poecke et al. (2001) demonstrated that *P. rapae* feeding but not mechanical damaged induced MeSA emissions. *P. xylostella* feeding also induced MeSA emission (Chen et al., 2003a). Chen et al. (2003) cloned a SAMT gene from Arabidopsis (*AtBSMT1*) and showed that it was inducible in leaves by wounding, *P. xylostella* and *F. occidentalis* herbivory, and MeJA but not SA treatment. It appears that although MeSA emissions are increased upon herbivory, this is not accompanied by increased SA levels or large change in SA-responsive gene-expression. This means either that salicylate biosynthesis is induced by herbivory and that the increased flux through this pathway is completely directed towards MeSA synthesis; or that herbivory-induced MeSA production only relies on stored SA (for example in the form of SA-glucoside).

3.2.3.3 Application. Many studies using SA application to study Arabidopsis-insect interactions have demonstrated a negative effect on JA-inducible defenses. For example, SA application interferes with *S. littoralis*-induced JA-levels (Stotz et al., 2002); with JA induced formation of trichomes (Traw and Bergelson, 2003); with the induction of indole glucosinolates and some aliphatic glucosinolates by MeJA in L*er* (Kliebenstein et al., 2002); and with the induction of total glucosinolates by JA in Ws-0 but not in Col-0 (Cipollini et al., 2004). Interestingly, some glucosinolates are induced by SA or the SA analogue INA and this induction can be inhibited by MeJA in both Col-0 and L*er* (Kliebenstein et al., 2002; Mikkelsen et al., 2003). Another glucosinolate is synergistically induced by JA and SA in L*er* (Kliebenstein et al., 2002). Similarly, Cipollini et al. (2004) showed synergistic effects of JA and SA on total glucosinolate levels in Col-0.

As SA clearly affects plant defenses, it is not surprizing that it also affects Arabidopsis insect interactions. SA treatment increased larval growth of *S. exigua* on Col-0 and Ws-0 (Cipollini et al., 2004), likely because it's inhibitory effect on JA-induced defense mechanisms such as glucosinolate accumulation. However, application of the SA-analogue BTH resulted in modestly reduced aphid fecundity (Moran and Thompson, 2001), suggesting that SA inducible defenses increase resistance against aphids. This is somewhat remarkable as JA defenses also increase resistance against aphids. Application of SA did not induce parasitoid attraction (Van Poecke and Dicke, 2002).

3.2.3.4 Mutants. Many mutants are available that interfere with SA signaling. One of the mutants discussed here, *sid2*, is a biosynthesis mutant impaired in the isochorismate pathway. Other mutants affect SA concentrations by being either positive or negative regulators of SA biosynthesis. The former include *eds1-2, eds5, eds15, pad4* and *mpk4,* the latter *cpr1, cpr6, hrl1* and *acd2.* There is also a transgenic plant, *NahG*, which contains a gene degrading SA. Besides mutants affecting biosynthesis, there is also a mutant that affects signaling downstream of SA: *npr1*. The effect of these different mutations on Arabidopsis-insect interactions is discussed next (see also figure 7).

3.2.3.4.1. *NahG*-degradation of SA. *NahG* transgenic plants contain a bacterial salicylate hydroxylase gene that converts SA into catechol (Delaney et al., 1994). Although this effectively removes endogenous SA, the degradation product, catechol, is known to cause side-effects (Heck et al., 2003). Therefore, the results using *NahG* plants may not reflect loss-of-SA responses but rather responses to catechol or a combination of both. Nevertheless, some results correspond nicely to results with SA application or other mutant plants. For example, *NahG* plants have higher basal levels of MeJA-inducible indole glucosinolates (Brader et al., 2001; Mikkelsen et al., 2003; Mewis et al., 2005) and higher levels of aphid and *S. exigua* induced aliphatic glucosinolates (Mewis et al., 2005). This corresponds nicely to alleviation of the inhibition of MeJA inducible indole glucosinolates by SA (Kliebenstein et al., 2002) and the higher basal levels of indole glucosinolates in other SA signaling impaired mutants (see below). *NahG* plants showed reduced larval growth of and defoliation by *T. ni* in both the Col-0 and L*er* background (Cui et al., 2002), which fits nicely with the reduced performance on other SA mutants and the increased performance of caterpillars on SA treated plants (Cipollini et al., 2004; and see below). As expected, *NahG* plants show an abolished emission of MeSA upon feeding by *P. rapae*. Additionally, these plants also show a strongly reduced sesquiterpene emission, which indicates a signaling role for (Me)SA in indirect defense, although an effect of catechol cannot be excluded (Van Poecke, 2002). The reduced emission of these volatiles is accompanied by a reduced attraction of parasitoid wasps by *NahG* plants (Van Poecke and Dicke, 2002).

3.2.3.4.2. *sid2, eds1-2, eds5, eds15* and *pad4* - interfering with SA biosynthesis. Most of the SA-pathway genes studied in Arabidopsis-insect interactions act upstream of SA accumulation, either through biosynthesis or regulatory functions. Several of the latter genes act as positive regulators of SA biosynthesis. Mutation of genes involved in biosynthesis or positive regulation interferes with SA accumulation. As such mutations have similar effects on plant-herbivore interactions, they are discussed together. The

SID2 gene encodes the enzyme isochorismate synthase and is part of SA biosynthesis. *SID2* is required for pathogen-induced production of SA. Mutation of SID2 affects both induced and, to a lesser extent, constitutive SA levels (Wildermuth et al., 2001). PAD4, EDS1 (from L*er* background) and EDS5 are positive regulators of SA signaling acting upstream of SA biosynthesis. PAD4 and EDS1 are homologous to triacyl glycerol lipases and EDS5 is homologous to MATE-transporters (Jirage et al., 1999; Nawrath et al., 2002). Just as *sid2, eds5* (also called *sid1*) has low levels of endogenous SA (free or bound by glucose) that cannot be induced by pathogens (Nawrath et al., 2002). Neither *EDS1* nor *PAD4* has reduced basal SA levels, but both show reduced accumulation of SA after infection by pathogens (reviewed by Wiermer et al., 2005). Other mutants that (may) interfere with SA signaling are *eds9-1* and *eds15*. However, these mutations have not been characterized and the corresponding genes not cloned. Thus, how these mutations affect SA signaling is unknown. The *eds15* mutant shows reduced SA accumulation upon pathogen infection, although less dramatic than the *sid2-2* mutant (Dewdney et al., 2000). The *eds9-1* mutant shows some similar, but not identical symptoms as *eds5* but its relationship with SA signaling is unclear (Rogers and Ausubel, 1997).

The *sid2, eds1-2,* and *pad4* mutants showed reduced growth of and defoliation by *T. ni, eds5* showed reduced growth of, but not defoliation by *T. ni*, and *eds15* did not show an effect on either *T. ni* growth or defoliation (Cui et al., 2002). Additionally, *eds5* and *eds9-1* showed no effect on aphid fecundity (Moran and Thompson, 2001). It should be noted that a gene-expression profiling study including *eds5, sid2-1, pad4, nrp1-1* (see below), *ein2-1* (ethylene signaling), and *coi1-1* (jasmonate signaling) demonstrated functions of PAD4 that appear to be independent of SA but dependent on JA/ET, whereas *eds5* and *sid2-1* only affect SA-signaling (Glazebrook et al., 2003), Although this may imply that some of the results of *pad4* are not due to effects on SA signaling, the results with sid2 indicate that the results of *pad4* can largely be explained through its effect on SA accumulation.

3.2.3.4.3. *cpr1, cpr6, hrl1* and *acd2* - constitutive activation of SA signaling. In contrast to the mutants described above, CPR1, CPR6, HRL1 and ACD2 are negative regulators of SA such that mutation of *CPR1, CPR6, HRL1* or *ACD2* results in elevated SA levels and SA-inducible responses (Bowling et al., 1994; Greenberg et al., 1994; Clarke et al., 1998; Devadas et al., 2002). For both CPR1 and 6 it has been demonstrated that they act upstream of PAD4, EDS1 and EDS5 (Clarke et al., 2000; Clarke et al., 2001; Jirage et al., 2001). *ACD2* encodes an enzyme thought to be involved in chlorophyll breakdown and mutation of this gene may lead to accumulations of breakdown products that trigger subsequent changes such as increased SA levels and lesion formation (Mach et al., 2001). Thus, ACD2 may only be indirectly involved in plant-defense signaling.

Corresponding to the enhanced SA-signaling, the *cpr1* mutant shows elevated levels of SA-inducible glucosinolates and reduced levels of some MeJA induced glucosinolates (Mikkelsen et al., 2003); and *hrl* showed reduced basal levels of both (insect-inducible) aliphatic and (MeJA-inducible) indole glucosinolates (Mewis et al., 2005). Additionally, the *hrl1* mutant showed reduced levels of *M. persicae* and *S. exigua* induced glucosinolates , but not of *B. brassicae* induced glucosinolates (Mewis et al., 2005).

The reduced levels of *hrl1* plant defenses correspond with an enhanced performance of *M. persicae*, but not of *B. brassicae* or *S. exigua.* Both *cpr* mutants and *acd2* showed enhanced growth of *T. ni*, which for *cpr6* and *acd2* corresponded with increased defoliation by this caterpillar (Cui et al., 2002). Again, these effects on Arabidopsis-insect interactions are likely caused by the interference with JA-induced defenses such as glucosinolate biosynthesis.

However, the effect of these mutants on herbivory and herbivory-induced responses may be more complex. It is known that both *cpr1* mutants and *hrl1* also show up-regulated JA/ET-inducible responses that are dependent on SA, JA and ET. Thus it was proposed that these mutants induce at least two SA-dependent pathways: a JA/ET-dependent pathway and a JA/ET-independent pathway (Clarke et al., 1998; Clarke et al., 2000;Devadas et al., 2002). How this relates to herbivore-induced responses is unclear.

3.2.3.4.4. *mpk4* - affecting JA/SA interactions. Another negative regulator of SA is MPK4, discussed previously. MPK4 mediated signaling is influenced by EDS1 and PAD4 and acts downstream of JA (Wiermer et al., 2005).The *mpk4* mutant shows a similar phenotype as the *cpr1* mutant with respect to glucosinolates, with the exception that MeJA cannot induce glucosinolates in this mutant at all (Mikkelsen et al., 2003). This latter difference between *cpr1* and *mpk4* may be caused by the additional role of MPK4 in jasmonate responses (see the discussion of *mpk4* in the jasmonate section).

3.2.3.4.5. *npr1* - affecting signal transduction downstream of SA. NPR1 (=NIM1) is an important component of signaling downstream of SA that is activated by an SA-induced change in redox-state of the cell. Many SA-inducible responses are blocked in the *npr1* mutant including SA-inhibition of JA-inducible responses. However, NPR1 is also required for some JA/ET responses. The different functions of NPR1 may be reflected in different subcellular localization, with transport of NPR1 to the nucleus being necessary for SA-dependent responses but not for JA/ET dependent responses, nor for the inhibition of JA/ET responses by SA (Glazebrook et al. 2003; and see Dong, 2004 for a review).

The *npr1-1*/*nim1* mutants (in Col-0 and Ws background respectively) showed enhanced basal levels of total glucosinolates (mainly aliphatic glucosinolates) (Cipollini et al., 2004; Mewis et al., 2005), but this mutation negatively affected JA-induced trichome formation (Traw and

Bergelson, 2003). The *npr1-1* mutants showed reduced growth of and defoliation by *T. ni,* reduced growth but not defoliation by *S. exigua* and reduced defoliation by *S. littoralis* (Stotz et al., 2002). The *nim1* mutant showed reduced growth and defoliation by *S. exigua* (Cipollini et al., 2004; Mewis et al., 2005). With respect to aphid fecundity, Mewis et al. (2005) reported reduced fecundity of both *M. persicae* and *B. brassicae* on *npr1-1*, although another study reported no effect of *npr1-1* on *M. persicae* fecundity (Moran and Thompson, 2001). No effect of *NPR1* mutation was found on P. rapae-induced parasitoid attraction (Van Poecke and Dicke, 2003). The same study that reported SA independent ET/JA dependent effects of *PAD4* mutation showed a similar effect of *NPR1* mutations. Thus, it is unclear whether the effects of *npr1-1* mutation on Arabidopsis-insect are due to interference with ET/JA signaling, SA signaling or both, although the results with other mutants show more similarity of *NPR1* mutation with deficiency in SA signaling

3.2.3.5 Interactions Between SA, JA and ET Pathways. From all these results, it becomes clear that SA, just like ET, mainly acts as a negative regulator of Arabidopsis defenses against herbivorous insects ranging from aphids to caterpillars. Inhibition of SA accumulation alleviates of inhibition of jasmonate responses. As a result, JA-inducible defenses such as glucosinolate production reach a higher level when the SA pathway is impaired. Thus, SA signaling deficient plants are generally more resistant against caterpillars. Mutations with a weaker effect on SA accumulation, such as *eds15*, also have a weaker effect on resistance. Inhibition of SA accumulations also enhances defense mechanisms upon aphid feeding; however, this does not seem to affect performance of the aphids. Mutations stimulating the SA pathway show enhanced inhibition of jasmonate responses and generally are less resistant to caterpillars and also to a generalist aphid. SA has a more positive effect on indirect plant defenses. Not only is MeSA an attractant of predators or parasitoids,it likely also has a signaling function as suggested by the lack of some terpenes in the headspace of caterpillar infested *nahG* plants.

Some of the components of the SA-signaling pathway are also involved in responses that are dependent on both JA and ET. For example, PAD4 and NPR1 are required for both SA as well as JA/ET dependent responses. Additionally, *cpr6* and *hrl1* mutants which show elevated levels of SA, also show up-regulated JA/ET-inducible responses. These local responses are dependent on SA, JA and ET and independent of NPR1. Thus it was proposed that these mutants induce at least two, SA-dependent pathways: a local, NPR1-independent, JA/ET-dependent pathway and a systemic, JA/ET-independent, NPR1-dependent pathway. Both pathways are demonstrated to be involved in respectively local and systemic resistance against biotrophic pathogens (Clarke et al., 1998; Clarke et al., 2000;Devadas et al., 2002). As both the SA and the JA/ET appear to work antagonistically to the 'JA only' pathway, PAD4, NPR1, CPR6 and HRL1 may negatively affect Arabidopsis defenses against insect herbivores in multiple ways (figure 7).

3.2.4 Abscisic Acid

In the paragraph on JA-ET interactions, it was mentioned that caterpillar feeding induced the JA/AtMYC pathway (which is either part of or identical to the 'JA only' pathway) and represses the JA/ET pathway possibly through concerted actions of JA and ABA. Thus, ABA may play a mediating role in directing signaling downstream of JA in an antagonistic manner to ET. Are there more indications that ABA plays a role in Arabidopsis-insect interactions? The short answer is no, not for Arabidopsis. However, loss of ABA did result in reduced resistance against caterpillars in tomato (Thaler and Bostock, 2004). ABA is mainly known as a dehydration stress hormone and it is also known to interfere with defenses against pathogens (Mauch-Mani and Mauch, 2005). Interestingly enough, feeding by *P. rapae* suppresses the expression of many dehydration-induced genes compared to wounding (Reymond et al., 2000) From this one might conclude that insect-feeding suppressed ABA induced responses, which is quite the opposite of what has been mentioned before. So far, there have been no reports that ABA levels increase upon herbivory. Whether and, if so, how ABA influences Arabidopsis-insect interactions is therefore unclear.

3.2.5 Gibberellins

Gibberellins are known for their role in developmental processes. So far, only one study demonstrated a possible effect of gibberellins on Arabidopsis defense against insects, as gibberellin induced trichome formation in new leaves L*er* synergistically with JA. This induction is inhibited by SA (Traw and Bergelson, 2003). However, as it is unknown whether herbivory induces gibberellin signaling, the relevance to inducible plant defense remains unclear.

3.2.6 Summarizing Signal Transduction

From all the information discussed, the picture arises of a signaling web where JA and other oxylipins play a central role, but where defenses can be fine-tuned by other hormones such as SA, ET and possibly others (figure 7). Jasmonates influence defense-responses to all kinds of insect herbivores, from phloem-feeders to tissue-munchers and from specialists to generalists. SA and ET mainly have an attenuating role on the induction of defenses against insect herbivores. Although these generalizations seem to be true for induction of defense responses, they may not be true for the effect on herbivore performance. It has been noted earlier that some defense responses, such as glucosinolate production, are mainly effective against generalists but not specialists. Similarly, ET seems to have a positive effect on the performance of generalists, likely at least partially due to reduced accumulation of JA-inducible glucosinolates caused by the antagonistic effects of ET,

but not on the performance of specialists. Studies on the effect of JA and SA on herbivore performance have almost exclusively focused on generalist herbivores. The only exception is a report of increased performance of *B. brassicae* on *coi1* mutants. It seems likely that performance of specialists will be less affected by induced defenses than performance of generalists.

An obvious question is why plants attenuate their inducible defenses by e.g. stimulating ET production upon herbivory? If this is merely a fine-tuning of defenses, one would expect that defenses should be optimized for specific attackers, which is obviously not the case as ET-signaling deficient mutants are more resistant. However, different combinations of signals induce different defenses. For example, although some defense responses will be down-regulated by the combination of JA and ET, others are up-regulated by this combination and yet others are up-regulated by JA irrespective of the presence of ET and so on. This is especially well-demonstrated with respect to induction of different glucosinolates. Thus, different combinations of signaling pathways induce different defense responses and these defense responses are effective against different kinds of attackers. Plants may not be able to defend themselves optimally to one kind of attacker (e.g. insect herbivores) without becoming more susceptible to other kinds of attackers (e.g. biotrophic pathogens). Such a trade-off between defenses could explain why plants are not optimally defended. The interactions between the effects of different attackers on plant defense are discussed next.

3.2.7 Responses to Multiple Attackers: Cross-Talk Among Signaling Pathways

Plant defense responses clearly depend on the type of attacker. These responses can roughly be divided in SA-dependent responses upon attack by biotrophic pathogens, JA/ET-dependent responses upon attack by necrotrophic pathogens, and 'JA-only' responses upon attack by insect herbivores. The tight and often negative interactions between different signaling pathways within the signaling web make it likely that the attack of one species, such as a SA inducing pathogen, will affect the plants responses to subsequent attacks by a next species, such as a caterpillar against which mainly JA-inducible defenses are effective. Indeed, several studies have investigated such interactions and found evidence for trade-offs. Cui et al. (2002) showed that previous infection of Arabidopsis with virulent *P. syringae* pv *maculicola* (*Psm*) resulted in increased performance of *T. ni* larvae. As virulent *P. syringae* mainly induces the SA-signaling pathway (Glazebrook, 2005), this seems to fit well with the model of antagonistic SA-JA interactions. Interestingly, although performance of *T. ni* is generally lower on SA-lacking genotypes such as *NahG* and *npr1*, virulent *P. syringae* still induced increased performance of *T. ni* in these mutants, indicating that this negative effect on Arabidopsis defenses is actually SA independent. This response was, however, dependent on PAD4. A possible explanation might by that *P. syringae*, known to induce beside SA, also JA and ET levels, elicits JA/ET dependent signaling. JA/ET signaling is known to work antagonistically to 'JA only' responses and is dependent on PAD4. It should be noted that this increased susceptibility seems a rather subtle effect, as researchers of the same group failed to reproduce these results later, possibly due to slightly different environmental conditions (Cui et al., 2005). Another unexpected result was that infection with avirulent *Psm* induced increased resistance to *T. ni* and increased susceptibility to *Psm* (Cui et al., 2002). This was subsequently demonstrated to be caused by the JA mimic coronatine, which is produced by these pathogens as a phytotoxin and likely aimed at reducing SA-dependent defenses which are effective against *Psm* (Cui et al., 2005). So, apparently there are two, counteracting processes at work. On the one hand, *Psm* induces a PAD4 dependent pathway that results in increased susceptibility to caterpillars. On the other hand, *Psm* induces a coronatine-dependent pathway that triggers JA-inducible defenses and results in increased resistance against caterpillars and susceptibility against *Psm* (Cui et al., 2005). To make matters even more complex, De Vos (2006) demonstrated that previous infestation with *P. rapae* induces resistance against avirulent *Pst*. This is rather unexpected as the work from Cui et al. (2005) demonstrated that *Psm* actually tries to undermine plant defenses by stimulating JA-responses. Similar to the results with virulent *Psm* and *T. ni,* the results reported by De Vos (2006) were independent of NPR1, SID2 or EDS5. However, they were also independent of JAR1, COI and EIN2, showing that neither SA, JA nor ET is likely to be involved. Additionally, De Vos (2006) showed that previous infection with *P. rapae* also induces resistance against turnip crinkle virus and the bacterial pathogen *Xanthomonas campestris* pv *armoraciae*, pathogens against which SA-inducible defenses are effective, but not against the fungal pathogen *Alternaria brassicicola*, against which JA/ET-inducible defenses are effective. It is also induced resistance against subsequent attack of *P. rapae* itself. The mechanisms behind these findings are unclear, but certainly reward more research.

Cleary, the effect of multiple attacks by different pathogens and pests influence each other in complex ways that may include the plant hormones SA, JA and ET, but also other, largely unknown pathways. This also might indicate why Arabidopsis apparently fails to optimally launch defenses against a certain attacker as discussed in the previous section: as multiple attacks by different species are likely to be common in the field, a plant optimizing its defenses against one pathogen or pest may leave it too vulnerable to attack by others.

4. EVOLUTION OF DEFENSES

Although in this text I have often used the word defense, actually the mechanisms discussed so far have mainly dealt with plant resistance. According to the definition of Rausher (1992) to qualify for the term "plant *defense*

mechanism against insects" such a mechanism should have been evolved or maintained within a plant population because of selective pressures exerted by insect herbivores, something that is not easily demonstrated. Arabidopsis is a very suitable model for studying evolution of plant defenses, not only because of its sequenced genome and other genetic and molecular resources, but also because of its wide geographical and environmental range (Mitchell-Olds, 2001). This means that there are many Arabidopsis populations that are growing in different environmental condition and therefore experiencing different selection pressures. Different ways to study evolution of defenses including phenotypic characterization of selection, genotypic characterization of selection, or a combination of both.

4.1 Phenotypic Characterization of Selection

Phenotypic characterization of selection often relies on correlations between a certain resistance trait and plant fitness. This illustrates a problem associated with phenotypic characterization of selection: correlations do not necessarily infer causal relationships. Nevertheless, phenotypic characterization of selection can be informative. However, most studies mentioned in this book chapter studied resistance by looking either at insect performance and/or level of inflicted damage (e.g. number of holes in leaves). Neither of these parameters necessarily affects plant fitness and therefore we do not know whether the mechanisms studied can be considered defense mechanisms. For example, a plant may be tolerant to herbivore damage, i.e. it may utilize its resources to compensate for herbivore damage rather to defend itself against herbivores. This may lead to situations where damaged plants are more fit than undamaged plants (Weinig et al., 2003).

4.1.1 Plant Fitness

Only a few studies did look at plant fitness. Examples include the positive effect of attracting parasitoids on fruit number and weight of total seed production in *P. rapae* infested Arabidopsis, but no evolutionary questions were addressed (van Loon et al., 2000).

In a field trial, Murren et al. (2005) found indications for a selection pressure on tolerance but not on resistance exerted by fungus gnats and aphids. However, there was no control treatment where herbivory was prevented, making these results hard to interpret. For example, herbivores may have had a preference for larger plants that before the onset of herbivory had a greater potential for seed production, resulting in a similar seed production by larger, infested plants and smaller, uninfested plants.

4.1.2 Effect of Glucosinolates, Trichomes and Tolerance on Plant Fitness

In an extensive field study, Mauricio and coworkers addressed several evolutionary questions with respect to the role of tolerance, glucosinolates and trichomes on fitness in natural populations of Arabidopsis in North Carolina. They concluded that in Arabidopsis: tolerance and resistance to insect herbivores are not mutually exclusive - a result confirmed also for herbivory by rabbits, although tolerance and resistance mechanisms are likely to differ between these studies (Weinig et al., 2003). Additionally, they concluded that herbivory exerts a stimulatory selective pressure on glucosinolate levels, trichome densities and tolerance; trichome density and glucosinolate levels are positively correlated; there is no correlation between the defense mechanisms on one hand and tolerance on the other; there are costs associated with both defense mechanisms and with tolerance; and a costs/benefit analyses resulted in a weak balancing selection for glucosinolates in the presence of herbivores, a disruptive selection on tolerance (directed either towards full tolerance or no tolerance, depending on the initial level of tolerance of the genotypes), and a negative selection pressure for trichome density (Mauricio and Rausher, 1997; Mauricio et al., 1997; Mauricio, 1998). The latter raises the question why trichomes are present in these populations. As glucosinolate levels and trichome densities are positively correlated, this may indicate genetic linkage between glucosinolate level and trichome traits, and such linkage disequilibrium can result in positive selection on glucosinolate levels that also increases trichome density. However, it could also be that trichome density is not at an evolutionary equilibrium in those populations and eventually trichomes may be eliminated (Mauricio and Rausher, 1997). The latter explanation demonstrates another problem associated with phenotypic characterization of selection: evolution takes place over long periods of time and a snapshot analyses may not reflect past selection pressures.

4.2 Genotypic Characterization of Selection

Genotypic characterization of selection provides us with a means to travel back in time. For example, by acquiring genetic information related to a certain resistance trait in many different genotypes (for example different accessions or different plant species), differences in nucleotide diversity can indicate adaptive evolution. This can be based on differences between the levels of non-synonymous mutations (resulting in an amino acid substitution) and synonymous mutations or on difference in the number of mutations between the gene(s) affecting the trait and their genetically linked regions on the one hand and regions undergoing neutral evolution on the other hand. Additionally, a difference in nucleotide diversity between duplicated genes within a genome (paralogues) can reveal the role of different evolutionary trajectories such as neutral drift, selective sweeps resulting in one allele becoming dominant (possibly reflecting an "evolutionary armsrace"), and balancing selections that allows for a diversity of alleles being present (possibly reflecting "trench warfare"), as has been done by sequence analyses of various Arabidopsis R-genes involved in gene-for-gene based

defense against pathogens (Stahl et al., 1999; Bergelson et al., 2001). These studies show the importance of gene-duplication in generating genes with novel functions (e.g. recognition of a different or an adapted pathogen), which may be especially important in biotic interactions such as between plants and pests. A disadvantage of the "characterizing of genotypic selection" method is that often there is only very limited information about the environmental conditions during evolution and therefore we cannot be sure about the identity of the selection pressures. Likely, the most reliable method combines information of genotypic variation in extant accessions and/or species with information on environmental parameters of the geographical origin of those genotypes (de Meaux and Mitchell-Olds, 2003), although again these environmental parameters may not reflect past selection pressures.

4.2.1 Evolution of Glucosinolate Biosynthesis and Degradation Genes

As mentioned in part II, dissection of glucosinolate biosynthesis and degradation pathways revealed a limited set of genes affecting elongation and modification of methionine side-chains (reviewed by Kliebenstein et al., 2005; see figure 4). For example, the *GS-ELONG* locus, involved in elongation of the methionine side-chains, consists of a three-member gene-family formed by gene-duplication and enzyme activity analysis shows functional divergence. MAM1 catalyzes two cycles of elongation, resulting in C4 side-chains, MAM2 catalyzes one cycle of elongation, resulting in C3 side-chains, and MAML catalyzes multiple cycles of elongation. Additionally, MAM2 activity was associated with higher total aliphatic glucosinolate. Sequence analyses revealed a balancing selection on *MAM2*, likely due to its impact on aliphatic glucosinolate level. The higher levels of aliphatic glucosinolates associated with *MAM2* confer resistance to the generalists *S. exigua* and *T. ni*, but not to the specialist *P. xylostella*. As there were no obvious costs to *MAM2* expression, the authors speculated that differences between the effects on generalist herbivores (demonstrated resistance) and specialists (possible attraction/feeding stimulation) may account for this balancing selection (Kroymann et al., 2003).

The *GS-AOP* locus is involved in side-chain modification. Sequence, gene-expression and biochemical analyses revealed three *AOP* genes and a pseudogene in three different alleles of the *GS-AOP* locus found in 21 Arabidopsis accessions (Kliebenstein et al., 2001c). Again, the AOP genes are the result of gene-duplication. Two genes *AOP2* and *AOP3* have been functionally characterized and produce alkenyl and hydroxyalkyl glucosinolates respectively. Interestingly, *AOP2* and *AOP3* expression appears to be mutually exclusive, which for *AOP2* is associated with polymorphisms in the coding region in a few accessions but with differences in gene-expression in others. Difference in *AOP3* activity is caused by differences in gene-expression rather than polymorphisms in the coding region. Variation in the *GS-AOP* locus thus resulted in three classes of accessions, one class that produces alkenyl glucosinolates associated with *AOP2* activity, one class that produces hydroxyalkyl glucosinolates associated with *AOP3* activity and one class that shows no side-chain modification associated with inactivity of both *AOP2* and *AOP3*.

A third locus is the eptihiospecifier *ESP* locus. As mentioned before, plants with a functional *ESP* gene degrade glucosinolates to nitriles rather than isothiocyanates. Sequence analyses revealed three classes of *ESP* alleles: functional alleles, non-functional alleles due to differences in gene-expression, and one non-functional allele due to a truncated protein. Nitriles are less effective in resistance against *T. ni*, and thus EPS activity seems to decrease rather than increase direct defenses (Lambrix et al., 2001). However, nitriles may attract parasitoids and thus contribute to indirect defenses (Van Poecke et al., 2001). Additionally, isothiocyanates are known attractants of specialist herbivores and producing nitriles instead of isothiocyanates may be a way to avoid detection by herbivores (Lambrix et al., 2001)

4.2.2 Evolution of Trichome Development and Proteinase Inhibitor Genes

The studies on glucosinolate production indicate the importance of gene duplication in evolution of plant defense mechanisms against herbivores. The importance of gene duplication is supported by studies on other defense mechanisms, such as proteinase inhibitors. A study by Clauss and Mitchell-Olds (2003) found indications of functional divergence between two paralogues of a trypsin inhibitor gene (*ATTI1* and *ATTI2*); extremely low levels of polymorphism for both; and indications of a selective sweep for *ATTI2*. An extension of this study found four additional *ATTI* genes in the same locus, one gene being inactive (Clauss and Mitchell-Olds, 2004). Again, indications of functional divergence was found (based on both amino-acid sequence and on transcriptional differences, which were correlated), although the genetic linkage between the genes is likely to hamper functional divergence (Clauss and Mitchell-Olds, 2004). In these studies, the effect of different paralogues on plant-insect interactions was not studied.

Trichome formation is another Arabidopsis insect-defense mechanism of which evolution was studied. Hauser et al. (2001) found two diverged sequence clades of the *GL1* locus. However, sequence diversity did not correlate to trichome densities and only weak evidence for deviation from neutrality was found.

Together, these studies have demonstrated the importance of duplication events on evolution of Arabidopsis defense traits related to plant-herbivore interactions, similar to plant-pathogen interactions. Moreover, indications for balancing selection and selective sweeps have been found. Not surprisingly, variation in traits can be caused by both differences in gene-regulation and in coding-sequence. The studies on evolution of glucosinolate defenses are particularly interesting as they include results

on the ecological effects and as such give an indication of which selection pressures may be involved. From this, it appears that specialist and generalist herbivores exert different, sometimes contrasting selection pressures on defense traits.

5. CONCLUSIONS

Most of this review has focused on the interaction between Arabidopsis and herbivorous insects. The results of recent years clearly show that Arabidopsis has an impressive range of defense mechanisms available. Many if not all of these mechanisms are inducible. The signaling network underlying this is highly complex with many signals interacting with each other. Illustrative of this is the diversity of oxylipin compounds present that each may have their own distinct role in defense; and the many different ethylene responsive elements found, some even having antagonistic effects to each other (McGrath et al., 2005). Add to this the many other signaling pathways, such as the salicylate and abscisic pathways and the complexity becomes clear. Each herbivore species may have a specific effect on this signaling network and these specific interactions may indicate both fine-tuned defense mechanisms that deal with specific attackers as well as attempts of the herbivore trying to disrupt these defense mechanisms. Fortunately, there are also indications of reduced complexity. For example, the plant's response to generalist and specialist caterpillars seems to be almost identical, indicating that it is the insect's response rather than the plant's response that results in the different interactions between Arabidopsis and these attackers. It will be interesting to see whether this also holds true for other insect species, with the possibility that differences in feeding mode have the most dramatic impact on induced defenses. However, the fact that different aphid species have slightly different effects on glucosinolate production warn us that these interactions may not be that simple.

One of the most impressive accomplishments is the tremendous improvement of our understanding of biochemical pathways of secondary metabolites. These advancements, together with our more detailed understanding of signal transduction provide us with a great opportunity to link the signaling network with the compounds that directly affect plant-insect interactions. Such an understanding will be pivotal to finding out how the different components affect plant-insect interactions and opens up the way to extensive hypothesis-driven assays in both the laboratory and the field.

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