

**Signal signature, transcriptomics,  
and effectiveness of  
induced pathogen and  
insect resistance in Arabidopsis**



# **Signal signature, transcriptomics, and effectiveness of induced pathogen and insect resistance in Arabidopsis**

**Signalen, transcriptomics en effectiviteit  
van geïnduceerde afweer tegen pathogenen  
en insecten in Arabidopsis**

(met een samenvatting in het Nederlands)

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*voor Inge, pa en ma!*



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# General introduction

## Demand for sustainable agriculture

With a world population of over 6 billion and an estimated growth of 2 billion before the year 2030 the pressure on the world's food supply is increasing rapidly. Agriculture is essential to meet the growing demand for sufficient food, feed and fiber. However, fertile lands to grow crops and natural resources for fertilization are rapidly declining. These problems will force mankind to develop alternative, more sustainable agricultural strategies. Moreover, crop production is threatened by various pests and diseases, which cause yield losses of 20–25% worldwide. Traditionally, farmers have selected crop plants with higher pest and disease resistance. By the time chemical pesticides became available, they enabled farmers to reduce yield losses of susceptible crops, but due to environmental and human health risks these chemicals are being progressively banned.

Biotechnology, particularly genetic engineering, might play an important role in keeping crop production effective, i.e. high production at low costs without unacceptable risks to the environment. For instance, crops that can grow under nutrient-poor conditions will increase the potential area for agriculture. Furthermore, insect- and disease-resistant crops will result in better yields in areas with high risks of attack.

So far, cultivation of transgenic crops is restricted to a few countries worldwide. Nevertheless, new transgenic products are becoming available on the agricultural market (James, 2004). For instance, genetically modified crops, such as cotton, maize, and soybean, which harbor a gene from the soil bacterium *Bacillus thuringiensis* that encodes a protein toxic to insects, are now being used worldwide. *Bt*-cotton, the most widely introduced transgenic species, makes up 15% of the global area used for cotton production. The *Bt*-protein is highly toxic to specific *Lepidoptera*, including the two major cotton pests *Pectinophora gossypiella* and *Helicoverpa zea* (James, 2002).

Although scientific research has shown that no short-term adverse effects of transgenic crops can be detected, public opinion is concerned about food safety and environmental consequences of these crops. Therefore, the risks of introducing new transgenic crops should be carefully evaluated and compared to side-effects of crops that become available through conventional breeding.

## **Plants in their environment: It is a hard life!**

Plants are sessile organisms that cannot flee when they become exposed to unfavorable conditions. From the moment its seeds germinate, plants are facing harmful abiotic conditions, such as drought, salinity, or flooding. Plants need to acquire nutrients for their growth, but are often confronted with a limited nutrient supply. In addition, plants are surrounded by potential harmful micro-organisms and herbivorous insects. To cope with these abiotic and biotic stresses plants need to adapt and defend themselves.

Most micro-organisms and insects are unable to successfully attack plants, because plants possess sophisticated mechanisms to resist attack by potential harmful organisms. For instance, possible invaders are resisted by extant defenses, e.g. a thick cell wall, cuticula or the presence of thorns, spikes or trichomes, which prevent the attacker from reaching the soft plant tissues. Plants may also contain high concentrations of toxic or repellent compounds. These types of physical or chemical barriers are collectively called non-host resistance. Pathogenic micro-organisms and herbivorous insects have evolved strategies to overcome the physical and chemical defenses that are constitutively deployed by their hosts. In such cases where attackers can invade the plant tissue and provoke disease, additional defense responses, called induced defenses, are necessary to restrict the attacker. A timely defense response is critical to minimize tissue damage and to ascertain plant survival upon attack.

Nowadays, *Arabidopsis thaliana* is used as a model species to investigate the mechanisms behind induced defenses against pests and diseases. This knowledge contributes to the development of good alternative strategies for improved crop protection. Applying innate plant defense responses in conventional breeding or biotech engineering will prove valuable in the establishment of sustainable agriculture.

## **Arabidopsis as a model for plant-pathogen and plant-insect interactions**

*Arabidopsis* is a member of the *Brassicaceae* (crucifer) plant family and is related to crop plants such as cabbage and mustard, which make up approximately 10% of the world's agricultural production. *Arabidopsis* has

several features that make it an excellent model plant. Firstly, it is easy to rear. Under controlled conditions plants can complete their life cycle in just 6 weeks. Moreover, it is small, does not require special growth conditions, and produces large amounts of seeds. Secondly, it has a relatively small diploid genome that was fully sequenced in the Arabidopsis Genome Initiative (Kaul *et al.*, 2000). Furthermore, Arabidopsis is easily manipulated by mutation or transformation. Hence, thousands of mutant and transgenic genotypes exist, most of which are T-DNA insertion lines generated by *Agrobacterium tumefaciens* infection of wild-type Arabidopsis plants (Alonso *et al.*, 2003; Tissier *et al.*, 1999). Thirdly, Arabidopsis is widely distributed, with accessions in Europe, Asia, North Africa and North America. This makes it possible to investigate natural variation in Arabidopsis.

Molecular plant biologists have adopted *A. thaliana* as a model in the 80's, but it did not enter the field of plant-pathogen interactions, because no pathogens were thought to be able to infect this cruciferous plant. This rapidly changed after the first discovery of Arabidopsis being a host for the downy mildew oomycete *Hyaloperonospora parasitica* (Koch and Slusarenko, 1990). Since then, surveys of pathogens and insects have provided a range of organisms that can attack or provoke disease under controlled laboratory conditions (Meyerowitz and Sommerville, 1994; Mitchell-Olds, 2001). Meanwhile, Arabidopsis has been shown to be susceptible to infection by various bacterial, fungal, oomycetous and viral pathogens. By the time that the genome sequence program for Arabidopsis had finished (Arabidopsis Genome Initiative), the first reports on the use of Arabidopsis as a host for insects were published. To date, several specialist and generalist herbivorous insects have been described to infest Arabidopsis. Arabidopsis responses to attack by phloem-feeding insects, such as aphids or whiteflies (Moran and Thompson, 2001), cell-content feeders, such as thrips and spider mites (Li *et al.*, 2002; Van Poecke *et al.*, 2003), and tissue-chewing insects from the class of *Lepidoptera*, such as the specialist caterpillar *Pieris rapae*, or the generalist species *Spodoptera exiqua* (Beet armyworm) or *Spodoptera littoralis* (Egyptian cotton leafworm) have now been studied (Reymond *et al.*, 2000; 2004; Stotz *et al.*, 2002).

Although Arabidopsis is an excellent model system for plant-microbe and plant-insect studies, it cannot be used as a model for all plant-microbe interactions. As a crucifer, this species is not able to establish a symbiosis with nitrogen-fixing Rhizobium bacteria or phosphate-acquiring mycorrhizal fungi. To study these interactions, other plants are adopted as model species, e.g. *Medicago truncatula* and *Lotus japonicus*. To extrapolate results obtained from Arabidopsis, plant-attacker interactions could also be studied in these additional model species.

## Inducible defense mechanisms against diseases and pests

### Mechanisms of induced resistance against pathogens

Plants possess a variety of inducible defense mechanisms to effectively combat invasion by microbial pathogens. Two types of induced defense against pathogens have been particularly well studied in *Arabidopsis*: systemic acquired resistance (SAR), induced upon infection with an avirulent pathogen (Ryals *et al.*, 1996) and rhizobacteria-mediated induced systemic resistance (ISR) (Pieterse *et al.*, 1996; 2002). Both induced defense mechanisms have been shown to be effective against a broad, although not identical, range of pathogenic micro-organisms, including oomycetous, fungal, bacterial, and viral pathogens (Pieterse *et al.*, 1996; Ton *et al.*, 2002; Van Wees *et al.*, 1997).

Signal transduction of rhizobacteria-mediated ISR was studied using the rhizobacterial strain *Pseudomonas fluorescens* WCS417r and the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pieterse *et al.*, 1996; 1998; Ton *et al.*, 2002). Expression of WCS417r-mediated ISR requires an effective jasmonate (JA) and ethylene (ET) response. In contrast to ISR, SAR is dependent on accumulation of salicylic acid (SA) (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). Both types of induced defense have been shown to require a functional regulatory protein NPR1, indicating that they share part of their signal transduction cascade (Pieterse *et al.*, 1998; Pieterse and Van Loon, 2004). While the induction of SAR is accompanied by a large transcriptional reprogramming, including the expression of many pathogenesis-related (*PR*) genes (Maleck *et al.*, 2000), ISR is not associated with increased expression of known defense-related genes (Van Wees *et al.*, 1999; Verhagen *et al.*, 2004). Instead, rhizobacteria-induced plant tissue responds faster and to a higher extent upon subsequent pathogen attack. This sensitization of the tissue is called “priming” for defense-related gene expression (Conrath *et al.*, 2002). The majority of the ISR-primed genes is JA-responsive (Verhagen *et al.*, 2004). The priming phenomenon of WCS417r-mediated ISR suggests a cost-efficient mechanism that does not involve large transcriptional reprogramming, but is quickly activated upon attack by a subsequent invader. Indeed, when induced defenses are activated only when needed, energy is saved for growth and reproduction under non-stressed conditions. This situation contrasts with e.g. SAR-expressing plants, where the constitutive expression of induced defenses is associated with impaired plant growth and lower fitness (Baldwin, 1998; Heidel and Baldwin, 2004; Heil *et al.*, 2000). In contrast, rhizobacteria-mediated ISR is often associated with promotion of plant growth in the absence of pathogens (Pieterse and Van Loon, 1999; Ryu *et al.*, 2004; Van Loon and Glick, 2004). Moreover, inducible defenses that are dependent on the accumulation of

particular defense-related proteins might be more vulnerable to adaptations of the attackers that circumvent these defenses. Priming for defense-related gene expression is unlikely to favor adaptation of the pathogen or insect and contributes to the durability of resistance traits.

### **Mechanisms of induced defenses against herbivorous insects**

Questions concerning induced defenses against insects were addressed originally from a more ecological point of view and received little attention from molecular biologists (Karban and Baldwin, 1997). With the development of several plant-insect systems in the 90's and the opportunity of molecular studies in *Arabidopsis*, data on induced defenses against insects are now becoming rapidly available.

In contrast to induced defense responses against microbial pathogens, induced defenses against insect attack are based on two strategies, direct and indirect defense. First, upon herbivore attack, plants mount direct defenses that target the attacker directly. This response includes the accumulation of toxic and repellent compounds, or decreased nutritional value of the leaf tissue, which affects the feeding behavior of the insect at the site of interaction (local induced resistance) or throughout the whole plant (systemic induced resistance).

The systemic response upon insect attack has been well studied in the tomato-*Manduca sexta* (tobacco hornworm) interaction and resembles the effect of wounding (Ryan, 2000). Upon wounding of tomato plants, a local and systemic accumulation of proteinase inhibitors (PIs) occurs. These PIs interfere with the digestive activity of the insect (Pearce *et al.*, 1991; Ryan, 1992). This direct systemic defense response is dependent on the release of an 18-amino acid peptide, called systemin, which was long thought to function as the long-distance signal (McGurl *et al.*, 1994). Additionally, Howe *et al.* (1996) found that JA is increased and required for the wound-induced defense response in tomato. Moreover, O'Donnell *et al.* (1996) showed that exogenous application of ethylene (ET) boosts the expression of PIs. In contrast, application of salicylic acid (SA) to tomato suppressed wound-inducible PI expression. Grafting experiments with tomato mutants defective in systemic wound signaling indicated that systemin functions near or at the wound site to trigger JA production which promotes the systemic defense response (Howe, 2005).

JA is likewise involved in the direct defense of *Arabidopsis*. JA-impaired mutants suffer from enhanced consumption by several insects, including several *Lepidoptera* species (McConn *et al.*, 1997; Reymond *et al.*, 2004; Stotz *et al.*, 2002). In general, the induced defense upon insect attack is dependent on increased JA production, but it is modulated by other stimuli, such as elicitors in the herbivore regurgitate and the plant hormone ABA (Peña-Cortés *et al.*, 1996).

Secondly, plants display an indirect defense response upon attack by herbivores. They deploy the help of carnivorous insects to reduce herbivory, by the production of a volatile odor blend that attracts natural enemies of the herbivore. Odor blends are highly plant-attacker specific and attract both predators and parasitoids. This indirect defense strategy results in reduced herbivore numbers (Dicke, 1999; Turlings *et al.*, 1990). As many of the carnivorous insects are specialists, the information from the odor source is highly regulated. Providing correct information to the predator or parasitoid enemy of the herbivorous insect must be an evolutionary trait, which is constantly modified as herbivores try to avoid detection or suppress volatile production (Karban and Baldwin, 1997; Pare and Tumlinson, 1999; Vet and Dicke, 1992).

Volatile production in plants can be triggered by wounding, herbivore feeding, or application of insect regurgitate. Elicitors from insect regurgitate, such as  $\beta$ -glucosidase, fatty acid-amino acid conjugates (i.e. volicitin from larvae of *S. exigua*), have been shown to trigger volatile production to a similar extent as the herbivore itself (Alborn *et al.*, 1997; Halitschke *et al.*, 2001; Mattiacci *et al.*, 1995), while mechanically wounded plants release only a subset of the compounds induced by herbivore feeding (Van Poecke *et al.*, 2001). A recent publication by Mithöffer *et al.* (2005) showed that continued mechanical damage of Lima bean leaves mimicked the volatile production of caterpillar-infested plants in a quantitative manner. Although continuous mechanical damage was shown to be sufficient to mimic volatile production upon insect feeding, it has not been demonstrated that direct defense responses are also triggered by continuous mechanical wounding. Moreover, it is possible that elicitors in oral secretions of the herbivore have an additional modulating role.

Recently, *Arabidopsis* was shown to be a good model to study the chemical responses in indirect defense against feeding by caterpillars. Van Poecke *et al.* (2001) were the first to show that feeding by the specialist caterpillar *P. rapae* on *Arabidopsis* induced volatile blends that attract the parasitoid wasp *Cotesia rubecula*. Furthermore, by analyzing transgenic *LOX2*-cosuppressed S12 plants, which can no longer accumulate JA upon mechanical wounding, it was shown that JA plays a key role in the indirect defense of *Arabidopsis* upon *P. rapae* feeding. Compared to wild-type *Arabidopsis* plants the attraction of the parasitoid wasp to *P. rapae*-damaged S12 plants was significantly reduced (Van Poecke and Dicke, 2002).

Some direct defenses seem to work adversely, i.e. decreased nutritional value of consumed plant tissue will lead to increased damage by the herbivore, which needs to consume more tissue for the same weight gain. It has been suggested that direct and indirect defenses could function additively in resisting an attacker. Kessler and Baldwin (2004) provided evidence for this hypothesis. A combination of insect growth-slowing direct, and predator-attracting indirect

defenses of *Nicotiana attenuata* resulted in additional mortality of *Manduca* larvae. As most herbivores are only susceptible to predation in their early larval stages, delayed development due to direct defense responses will increase the chance of successful indirect defense.

As specialist herbivores complete their life cycle on one or a few related plant species, it has been hypothesized that continuing co-evolution has provided them with mechanisms to avoid recognition by the host plant. Moreover, the evolutionary arms race between plant and attacker might result in detoxification mechanisms in the insect. For example, toxic glucosinolates from cruciferous plants are detoxified by *P. rapae* (Wittstock *et al.*, 2004). In addition, insect-derived compounds, such as regurgitate proteins, can modulate or interfere with expression of host defenses (Wittstock *et al.*, 2004). It is thought that generalist caterpillars are not able to deploy these mechanisms, because they feed on a broad range of host plants and, thereby, encounter multiple plant species-specific defense strategies.

## Signal transduction in plant-attacker interactions

Effective resistance mechanisms are dependent on a fast recognition of the attacker encountered. Upon perception and recognition of attacker-derived elicitors plants need to process this information in order to mount local and systemic defensive responses. To transform the perception of environmental or attacker-specific stimuli into an appropriate defense response, signal transduction pathways involving SA, JA and ET have proven to be of major importance. SA, JA, and ET accumulate to varying levels in response to pathogen infection or damage caused by insect feeding, resulting in the activation of distinct sets of defense-related genes (Glazebrook *et al.*, 2003; Maleck *et al.*, 2000; Reymond *et al.*, 2004; Schenk *et al.*, 2000; Van Wees *et al.*, 2003). Compelling evidence for the significance of SA, JA, and ET in primary defenses came from studies with mutant and transgenic plants affected in either SA, JA, or ET synthesis or signaling (Pieterse *et al.*, 2001). For instance, SA-defective signaling mutants and transgenics are more susceptible to biotrophic pathogens than wild-type plants (Delaney *et al.*, 1994; Nawrath and Métraux, 1999; Wildermuth *et al.*, 2001). Blocking the response to JA generally renders plants more susceptible to herbivorous insects (Howe *et al.*, 1996; Kessler and Baldwin, 2004; Li *et al.*, 2002; 2003; McConn *et al.*, 1997). Enhanced susceptibility towards necrotrophic pathogens has been reported as well (Staswick *et al.*, 1998; Thomma *et al.*, 1998). Furthermore, analysis of mutants affected in ET signaling demonstrated that ET plays a modulating role in many plant defense responses (Hoffman *et al.*, 1999; Knoester *et al.*, 1998; Lund *et al.*, 1998; O'Donnell *et al.*, 1996).

Other plant signaling molecules such as abscisic acid (ABA) (Audenaert *et al.*, 2002; Ton and Mauch-Mani, 2004), and brassinosteroids (Goda *et al.*, 2004; Mussig *et al.*, 2002; Nakashita *et al.*, 2003) have recently been shown to also contribute to defense against pathogens and insects. These hormones possibly influence disease resistance by modulating or interfering with resistance signal transduction pathways. Anderson *et al.* (2004) clearly showed that high levels of ABA can suppress JA/ET-dependent expression of the *PLANT DEFENSIN1.2* (*PDF1.2*) gene. Moreover, mutants impaired in ABA synthesis (*aba2-1*) showed increased *PDF1.2* expression and higher resistance against the fungal wilt pathogen *Fusarium oxysporum*. A positive regulator of ABA signaling, the transcription factor AtMYC2, was shown to play a role in the interaction between ABA and JA (Anderson *et al.*, 2004).

### **Signal pathway cross-talk: a regulatory potential for optimizing inducible defenses**

While the importance of SA, JA, and ET in induced plant defense is clear, evidence is accumulating that their signaling pathways cross-communicate (Bostock, 2005; Dicke and Van Poecke, 2002; Felton and Korth, 2000; Feys and Parker, 2000; Kunkel and Brooks, 2002; Pieterse *et al.*, 2001; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998; Rojo *et al.*, 2003; Van Poecke and Dicke, 2002). For instance, activation of SA-dependent SAR has been shown to suppress JA signaling in plants, thereby prioritizing SA-dependent resistance to microbial pathogens over JA-dependent defense, which is, in general, more effective against insect herbivory (Bostock, 1999; Felton and Korth, 2000; Stout *et al.*, 1999; Thaler *et al.*, 1999; 2002). Pharmacological and genetic experiments have indicated that SA-mediated suppression of JA-inducible gene expression plays an important role in this process (Doares *et al.*, 1995; Doherty *et al.*, 1988; Glazebrook *et al.*, 2003; Gupta *et al.*, 2000; Harms *et al.*, 1998; Peña-Cortés *et al.*, 1993; Van Wees *et al.*, 1999). The antagonistic effect of SA on JA signaling was recently shown to be controlled by a novel function of the defense regulatory protein NPR1 in the cytosol (Spoel *et al.*, 2003). The site of action where SA-activated NPR1 exerts its antagonizing effect on the JA signaling pathways is currently unknown (Pieterse and Van Loon, 2004). Others, (Niki *et al.*, 1998), have shown that there are mutual antagonistic effects between SA and JA signaling, implying that a higher JA production can also reduce SA accumulation and action. Thus, plants are able to utilize cross-talk as a way to prioritize one defense pathway over the other in order to display the appropriate response to the attacker encountered.

Just as *P. rapae* takes advantage of the presence of toxic glucosinolates, various pathogens can modulate plant signal transduction for their own benefit

by taking advantage of the cross-talk between defense signaling pathways. Kloeck *et al.* (2001) showed that the *Pseudomonas syringae*-derived JA-mimicking phytotoxin, coronatine (COR), acts to promote disease by suppressing SA-dependent defenses. Using both wild-type and coronatine-insensitive *jai1* tomato plants and wild-type and COR-non-producing *P. syringae* pv. *tomato* bacteria, Zhao *et al.* (2003) demonstrated that the causal agent of bacterial speck disease activates the JA signaling pathway to actively suppress the SA-dependent defenses deployed by the host plant. In addition, COR and MeJA were shown to induce systemic susceptibility to *P. syringae* pv. *maculicola*. Pathogen proliferation was promoted in distal (untreated) tissues after application of these chemicals (Cui *et al.*, 2005). However, Block *et al.* (2005) showed that in Arabidopsis COR is an important virulence factor for *P. syringae* pv. *tomato* infection, but that its action is independent of SA suppression, as coronatine-compromised *P. syringae* pv. *tomato* was more virulent on SA-impaired plants compared to susceptible control plants. Furthermore, Rao Uppalapati *et al.* (2005) showed that exogenously applied COR and MeJA both induced JA-responsive gene expression in tomato, but with only a limited overlap in the transcriptome profiles. These data indicate that, although COR mimics JA action, both compounds affect host gene expression differentially.

These results imply that co-evolution between the host plant and its pest or disease agents allows attackers to manipulate plants for their own benefit by shutting down induced defense through interference with the defense signaling network (Kahl *et al.*, 2000; Zhao *et al.*, 2003).

## **Cross-talk between resistance against insects and pathogens**

SA, JA, and ET evidently play an important role in induced defenses against both microbial pathogens and herbivorous insects. As a consequence, effects of cross-talk between signal transduction pathways at the level of resistance against pathogens and insects are to be expected. For example, Felton *et al.* (1999) demonstrated that transgenic tobacco plants with reduced SA levels exhibited enhanced herbivore-induced resistance to *Heliothis virescens* (tobacco budworm) larvae. Conversely, SA-overproducing tobacco plants showed a constitutive induction of SAR that coincided with a strong reduction of herbivore-induced insect resistance.

Application of chemical SAR inducers, such as benzothiadiazole (BTH) or 2,6-dichloroisonicotinic acid (INA), has been shown to negatively affect insect resistance as well. For example, BTH induced resistance in tomato against *P. syringae* pv. *tomato*, but improved suitability of tomato leaves for feeding by leaf-chewing larvae of the corn earworm, *Helicoverpa zea* (Stout *et al.*, 1999).

A similar phenomenon was observed by Thaler *et al.* (1999), who showed that application of BTH to field-grown tomato plants compromised resistance to the beet armyworm. Moreover, development of the same insect was shown to be faster on *Arabidopsis* plants treated with SA, or on transgenic plants with elevated SA levels (Cipollini *et al.*, 2004). The cabbage looper, *Trichoplusia ni*, was shown to be reduced in weight gain when feeding on SA-impaired mutants compared to wild-type control plants (Cui *et al.*, 2002). Conversely, a faster development was observed in plants treated with SA (Cui *et al.*, 2005). In most cases, reduced insect resistance observed in plants treated with SA or its chemical analogue, can be attributed to the inhibition of JA action by increased SA levels.

Cross-talk between signal transduction pathways gives plants the possibility to prioritize a particular signal transduction pathway over the other in order to fine-tune the defense responses required to arrest invasion. In this respect, several important questions remain unanswered: What responses are triggered when plants are attacked simultaneously by multiple invaders? How do these responses interact? And will these responses lead to protection to a broad range of possible invaders, or induce susceptibility because the defenses have been optimized to arrest the first attacker encountered? Experiments to address these questions are limited due to the difficulties in experimental set-up and problems to distinguish which plant responses are corresponding to each of the attackers used.

The spectrum of effectiveness against different pathogens of two well-studied biologically induced defense mechanisms has been studied in *Arabidopsis*. Biologically induced SAR is associated with defenses against pathogens that are arrested by SA-dependent defense responses, while pathogens solely resisted by JA-dependent defense responses were not affected by induction of SAR (Ton *et al.*, 2002). In addition, *P. fluorescens* WCS417r-mediated induced resistance confers resistance only to pathogens that are sensitive to JA/ET-dependent defenses. A combination of both ISR and SAR showed additive resistance against the leaf pathogen *P. syringae* pv. *tomato* (Van Wees *et al.*, 2000) and against other pathogens restricted by both mechanisms (Pieterse and Van Pelt, unpublished data).

Recently, the effectiveness of SAR and ISR against herbivorous insects with different feeding strategies has been put under investigation. Van Oosten *et al.* (unpublished results) found evidence that development of some, but not all herbivores is affected by WCS417r-mediated ISR. In contrast to *P. rapae*, the generalist caterpillar, *S. exigua*, displayed decreased development on ISR-expressing plants. Moreover, when SAR was induced by an avirulent strain of *P. syringae*, larval development of both *P. rapae* and *S. exigua* was reduced. These findings contrast with the effect of exogenously applied SA, but can be

explained by the increased levels of JA and induction of JA-responsive gene expression in SAR triggered by *P. syringae* pv. *tomato* (Pieterse *et al.*, 2000).

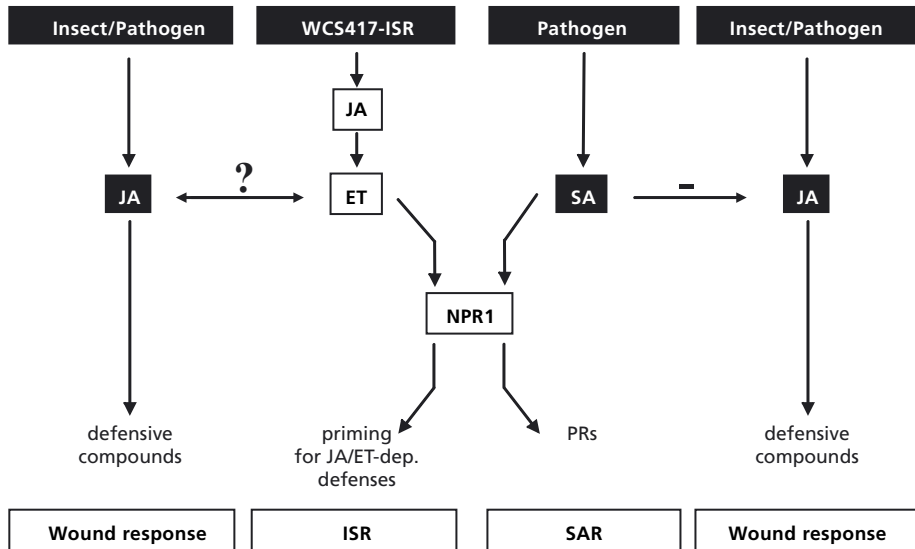
Several further studies have shown that activation of particular defense pathways negatively affects resistance to a subsequent attack by pathogens or insects. For instance, Moran (1998) demonstrated that in cucumber plants pathogen-induced SAR against the fungus *Colletotrichum orbiculare* was associated with increased feeding by the spotted cucumber beetle, *Diabrotica undecimpunctata*, and with enhanced reproduction of melon aphids (*Aphis gossypii*). A similar phenomenon was observed by Preston *et al.* (1999), who demonstrated that tobacco mosaic virus (TMV)-inoculated tobacco plants were more suitable for grazing by larvae of the tobacco hornworm (*Manduca sexta*) than non-induced control plants. Furthermore, infection of plants by rust fungi is well-known to influence herbivore behavior (Rostas *et al.*, 2003). The tripartite interaction between the leaf beetle *Gastrophysa viridula*, the biotrophic rust fungus *Uromyces rumicis*, and their common host plant *Rumex* has been intensively studied. Hatcher *et al.* (1994) have shown that adult beetles prefer feeding on healthy plants. Moreover, oviposition behavior was negatively influenced by rust infection. In contrast, peanut plants infected by white mold (*Sclerotium rolfsii*) were consumed to a larger extent by *S. exigua* (Cardoza *et al.*, 2002). Caterpillar performance was not studied in this interaction, and increased grazing might be due to a decreased nutritional value of the tissue. Moreover, the mechanisms behind these cross-resistances between pathogens and insects remain unclear.

Recently, Kessler and Baldwin (2004) reported on naturally occurring protection in *Nicotiana attenuata*. Previous infestation by the mirid bug, *Tupiocoris notatus*, induced direct and indirect defenses, decreasing development of the tobacco hornworm. Plants infested with the mirid bug were believed to have a significant fitness advantage in natural habitats where both herbivores are present, particularly when adult hornworm moths avoid oviposition on mirid-infested plants.

Understanding the plant's responses to insect attack will lead to knowledge that could be of use for crop protection. Indeed, understanding the underlying molecular mechanisms leading to cross-resistance occurring as a result of multiple, simultaneous, or consecutive pathogen or insect attacks might provide possibilities for novel approaches to improve plant resistance to natural pests and diseases.

## Outline of the thesis

The experimental part of this thesis starts off by studying the response of *Arabidopsis* to several attackers, including microbial pathogens and herbivorous



**Figure 1.** Proposed models for signaling pathways in non-pathogenic *Pseudomonas fluorescens* WCS417r-mediated ISR, pathogen-induced SAR, and insect-induced wound responses. SAR partly overlaps with the signaling pathway of ISR and is known to antagonize the wound response. Cross-talk between ISR and the wound response has not been investigated previously. SA, JA, and ET are involved in the induced defense against insects and pathogens. Increases in signaling compound levels are indicated by filled boxes, while open boxes, indicate compounds that are required for induction of resistance but do not increase in concentrations. For convenience, the wound response is depicted twice to allow cross-talk to be visualized.

insects with distinct feeding strategies. In Chapter 2 it is shown that Arabidopsis responds differentially to each attacker. In each combination we determined levels of SA, JA, and ET (signal signature) and correlated these with transcriptome changes. Plants attacked by the necrotrophic fungal pathogen *Alternaria brassicicola*, tissue-chewing larvae of *P. rapae*, or cell-content feeding thrips (*Frankliniella occidentalis*) all contained increased levels of JA. Moreover, a high percentage (44–69%) of all genes that were differentially expressed in these three interactions, were also responsive to MeJA treatment. However, only a limited overlap between JA-responsive attacker-induced genes was observed. This suggests that, although JA is the primary signal, other factors are involved in triggering this highly attacker-specific gene expression.

In Chapter 3 cross-resistance between insects and microbial pathogens is investigated. Will the transcriptional reprogramming upon *P. rapae* feeding eventually lead to resistance against future caterpillar attack? And is this type of resistance only effective against the same attacker or does it confer resistance to a wider spectrum of attackers? To address these questions, a bioassay was developed in which plants were pre-treated with *P. rapae* and subsequently challenge inoculated with an attacker of choice. In Arabidopsis, *P. rapae* and a factor in its regurgitate triggered responses conferring effective protection against

the caterpillar itself. Furthermore, this induced resistance was effective against some, but not all microbial pathogens tested. Although sensitive to JA-induced responses, a subsequent infection by the fungal pathogen *A. brassicicola* was not affected by prior herbivore feeding. Elicitors in the caterpillar regurgitate appeared to actively suppress a branch of the JA response that is involved in pathogen resistance (exemplified by *PDF1.2* expression), thereby antagonizing JA-dependent defenses against *A. brassicicola*, and prioritizing JA-dependent defense against insect feeding. *P. rapae* feeding did result in local, but not systemic, resistance against two leaf bacteria, *P. syringae* and *Xanthomonas campestris*. Infection by turnip crinkle virus, which is only arrested by SA-dependent defense responses, was reduced both locally and systemically by prior caterpillar feeding. Further analysis suggests that priming of SA-regulated defense pathways as a result of *P. rapae* feeding is involved.

Chapter 4 addresses the continuing evolutionary arms race between the host plant and its attackers. It is shown that a factor present in *P. rapae* regurgitate is involved in the suppression of host defense-related genes. *PDF1.2* expression, which is quickly induced upon mechanical damage, was shown to be suppressed by insect feeding or application of its regurgitate to wounded leaves. As *PDF1.2* expression is concomitantly regulated by the action of JA and ET, we investigated whether other well-known JA-regulated genes were also down-regulated by *P. rapae* or application of its regurgitate. The mRNA levels of *VEGETATIVE STORAGE PROTEIN2 (VSP2)*, *JASMONATE METHYL TRANSFERASE (JMT)*, and *LIPOXYGENASE2 (LOX2)* were not affected in a similar manner as *PDF1.2*. To study the involvement of ET, its accumulation in *P. rapae*-attacked, mechanically damaged, or regurgitate-treated plants was determined. Wounded leaves treated with caterpillar regurgitate or a water control showed similar ET accumulation, ruling out a role for reduced ET accumulation in the suppression of host defenses by *P. rapae* feeding. To identify genes that are similarly suppressed by *P. rapae* or its regurgitate, existing Affymetrix ATH1 microarray data sets were analyzed. Several genes showed similar induction patterns as *PDF1.2* in *P. rapae* and regurgitate-treated leaves pointing to a specific suppression of a subset of JA-inducible genes. Thus, the specialist caterpillar, *P. rapae* is able to interfere with the host defense mechanism and factor(s) in its regurgitate are important in this suppression. The JA-responsive transcription factor *AtMYC2* was identified to play a role in this insect-induced suppression of a branch of the JA signaling pathway.

Chapter 5 discusses the involvement of the transcription factor *AtMYB102* in resistance against *P. rapae*. *AtMYB102* gene expression was shown previously to be induced upon wounding and dehydration, as well as after treatment with ABA and JA (Denekamp and Smeekens, 2003). Micro-

array data showed that *AtMYB102* is up-regulated early after feeding by larvae of the white cabbage butterfly. Moreover, reporter line studies of a translational fusion between the promoter of *AtMYB102* and the  $\beta$ -glucuronidase open reading frame showed a clear activation of *AtMYB102* at the feeding sites of *P. rapae*. While caterpillar growth was faster on *myb102* T-DNA knockout lines compared to Col-0 wild-type plants, *AtMYB102* over-expressing plants did not show a reduced caterpillar development. This suggests that basal expression of *AtMYB102* contributes to resistance against *P. rapae*, while induced over-expression does not increase caterpillar resistance further. Transcription factors of the R2R3-MYB family have been implicated in many processes, including development and defense (Mengiste *et al.*, 2003; Stracke *et al.*, 2001; Verhagen, 2004). To identify genes controlled by the *AtMYB102* transcription factor, a micro-array study was performed to compare the expression of approximately 6000 Arabidopsis genes in wild-type Col-0 and *AtMYB102* over-expressing plants. Results obtained showed increased expression of many genes with a function in metabolism (20%), while 10% were stress-responsive. Genes that were induced in the over-expressing plants compared to the control were mostly localized to the cell wall and plasma membrane (42% and 17%, respectively). Based on these results it is tempting to speculate that *AtMYB102* plays a role in cell wall repair or strengthening upon caterpillar feeding. Finally, all results described in this thesis are discussed in Chapter 6. The data are evaluated critically with reference to current views on plant-pathogen and plant-insect interactions.

# Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack

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## Abstract

Plant defenses against pathogens and insects are regulated differentially by cross-communicating signaling pathways in which salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play key roles. To understand how plants integrate pathogen- and insect-induced signals into specific defense responses, we monitored the dynamics of SA, JA, and ET signaling in *Arabidopsis* after attack by a set of microbial pathogens and herbivorous insects with different modes of attack. *Arabidopsis* plants were exposed to a pathogenic leaf bacterium (*Pseudomonas syringae* pv. *tomato*), a pathogenic leaf fungus (*Alternaria brassicicola*), tissue-chewing caterpillars (*Pieris rapae*), cell-content-feeding thrips (*Frankliniella occidentalis*), or phloem-feeding aphids (*Myzus persicae*). Monitoring the signal signature in each plant-attacker combination showed that the kinetics of SA, JA, and ET production varies greatly in both quantity and timing. Analysis of global gene expression profiles demonstrated that the signal signature characteristic of each *Arabidopsis*-attacker combination is orchestrated into a surprisingly complex set of transcriptional alterations in which, in all cases, stress-related genes are overrepresented. Comparison of the transcript profiles revealed that consistent changes induced by pathogens and insects with very different modes of attack can show considerable overlap. Of all consistent changes induced by *A. brassicicola*, *P. rapae*, and *F. occidentalis*, more than 50% were also induced consistently by *P. syringae*. Notably, although these four attackers all stimulated JA biosynthesis, the majority of the changes in JA-responsive gene expression were attacker-specific. All together our study shows that SA, JA, and ET play a primary role in the orchestration of the plant's defense response, but other regulatory mechanisms, such as pathway cross-talk or additional attacker-induced signals, eventually shape the highly complex attacker-specific defense response.

## Introduction

Plants are abundantly present on earth and are at the basis of almost all food webs. Each of the approximately 300.000 plant species is attacked by a multitude of other organisms such as insects and pathogens. The number of insect species is estimated to be in the order of 6 million, 50% of which are herbivorous (Schoonhoven *et al.*, 1998). The biodiversity of pathogenic microorganisms is less well characterized but it is general knowledge that plant pathogens are a common threat to plants. To effectively combat invasion by microbial pathogens and herbivorous insects, plants have evolved sophisticated defensive strategies to “perceive” attack by pathogens and insects, and to

translate this “perception” into an appropriate defensive response (Dangl and Jones, 2001; Dicke and Hilker, 2003; Pieterse and Van Loon, 2004). These induced defense responses are regulated by a network of interconnecting signal transduction pathways in which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play key roles (Dicke and Van Poecke, 2002; Glazebrook, 2001; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998; Thomma *et al.*, 2001). SA, JA, and ET accumulate in response to pathogen infection or damage caused by insect feeding, resulting in the activation of distinct sets of defense-related genes (Glazebrook *et al.*, 2003; Reymond *et al.*, 2004; Schenk *et al.*, 2000). Compelling evidence for the significance of SA, JA, and ET in plant defense came from studies using mutant and transgenic plants affected in either SA, JA, or ET signaling (reviewed in Pieterse *et al.*, 2001; Pozo *et al.*, 2005). For instance, SA-defective signaling mutants and transgenics are often more susceptible to pathogen infection than wild-type plants (Delaney *et al.*, 1994; Nawrath and Métraux, 1999; Wildermuth *et al.*, 2001). Blocking the response to JA generally renders plants more susceptible to herbivorous insects (Howe *et al.*, 1996; Kessler *et al.*, 2004; McConn *et al.*, 1997), although enhanced susceptibility towards necrotrophic pathogens has been reported as well (Staswick *et al.*, 1998; Thomma *et al.*, 1998). Furthermore, analysis of mutants affected in ET signaling demonstrated that ET plays a modulating role in many plant defense responses (Hoffman *et al.*, 1999; Knoester *et al.*, 1998; Lund *et al.*, 1998).

While the importance of SA, JA, and ET in induced plant defense is clear, evidence is accumulating that their signaling pathways cross-communicate (Dicke and Van Poecke, 2002; Felton and Korth, 2000; Feys and Parker, 2000; Kunkel and Brooks, 2002; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998; Rojo *et al.*, 2003). For instance, activation of SA-dependent systemic acquired resistance (SAR) has been shown to suppress JA signaling in plants, thereby prioritizing SA-dependent resistance to microbial pathogens over JA-dependent defense that is, in general, more effective against insect herbivory (Felton and Korth, 2000; Stout *et al.*, 1999; Thaler *et al.*, 1999; 2002). Pharmacological and genetic experiments have indicated that SA-mediated suppression of JA-inducible gene expression plays an important role in this process (Glazebrook *et al.*, 2003; Peña-Cortés *et al.*, 1993; Van Wees *et al.*, 1999), and can sometimes work in both directions (Glazebrook *et al.*, 2003; Niki *et al.*, 1998). The antagonistic effect of SA on JA signaling was recently shown to be controlled by a novel function of the defense regulatory protein NPR1 in the cytosol (Pieterse and Van Loon, 2004; Spoel *et al.*, 2003). Cross-talk between defense signaling pathways is thought to provide the plant with a powerful regulatory potential, which helps the plant to “decide” which defensive strategy to follow, depending on the type of attacker it is encountering. Yet, it

may also allow attackers to manipulate plants to their own benefit by shutting down induced defense through influences on the signaling network (Kahl *et al.*, 2000).

In order to study the role of pathway cross-talk in plant innate immunity, it is important to have insight into the dynamics of SA-, JA-, and ET-signaling during different plant-attacker combinations. The role of SA, JA, and ET in plant defense has been studied for several plant-microbe and plant-insect interactions (Dicke and Van Poecke, 2002; Glazebrook, 2001; Pieterse *et al.*, 2001). However, most of these studies have been performed in different plant species, often using single plant-microbe or plant-insect combinations. Moreover, the large variation in experimental conditions in these studies makes it difficult to integrate the results and draw overall conclusions. Therefore, we monitored the dynamics of SA-, JA-, and ET-signaling in a single plant species (*Arabidopsis thaliana*) in response to attack by a range of microbial pathogens and herbivorous insects with very different modes of action. To relate our findings to those by others, we investigated the response of *Arabidopsis* to the well-characterized microbial pathogens *Pseudomonas syringae* pv. *tomato* and *Alternaria brassicicola* and the herbivorous insects *Pieris rapae*, *Myzus persicae*, and *Frankliniella occidentalis*. The production of SA, JA, and ET was monitored during these five *Arabidopsis*-attacker interactions, and related to global gene expression profiles using Affymetrix ATH1 whole-genome GeneChips.

## Results

### **Arabidopsis pathogens and insects**

*Arabidopsis* has been proven to be an excellent model for studying a wide variety of plant-pathogen and plant-insect interactions (Kunkel, 1996; Van Poecke and Dicke, 2004). To study the dynamics of the response of *Arabidopsis* to different microbial pathogens and herbivorous insects simultaneously, we chose two well-characterized *Arabidopsis*-pathogen interactions and three *Arabidopsis*-insect interactions in which the attackers deploy very different modes of attack.

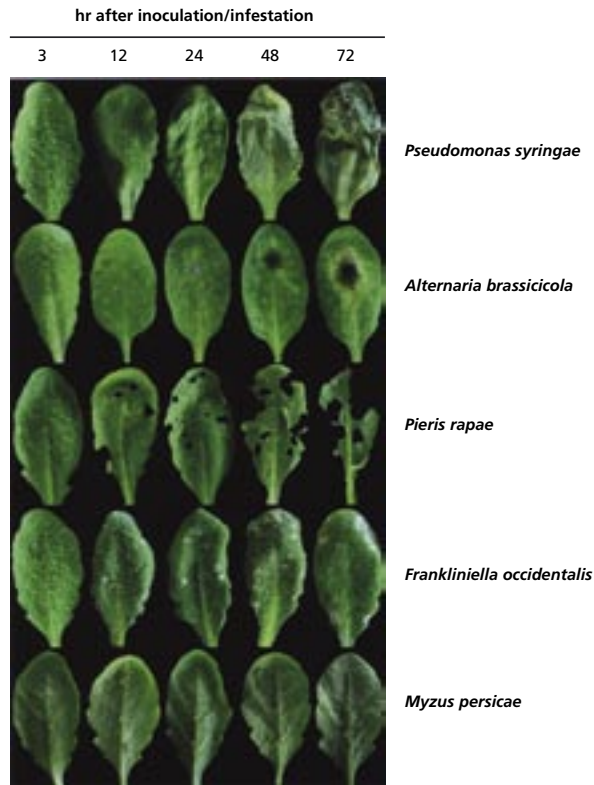
*P. syringae* is a bacterial leaf pathogen that causes extensive chlorosis and necrotic spots (Whalen *et al.*, 1991). Analyses of *Arabidopsis* signaling mutants have shown that basal resistance to this pathogen is predominantly dependent on SA (Delaney *et al.*, 1994; Nawrath and Métraux, 1999; Wildermuth *et al.*, 2001), although components of the JA and ET signaling pathways have been demonstrated to contribute to resistance against this pathogen as well (Ellis *et al.*, 2002; Pieterse *et al.*, 1998). The transcriptome of *Arabidopsis* in response to *P. syringae* pv. *maculicola* infection has been well-studied (Glazebrook *et al.*,

2003). Recently, Tao *et al.* (2003) provided evidence that a large part of the differences in transcriptional changes between the compatible and incompatible interactions is quantitative. Therefore, to induce a strong response in the plant, we chose to use avirulent *P. syringae* pv. *tomato* DC3000, carrying the avirulence gene *avrRpt2*. Pressure infiltration of whole Arabidopsis leaves with *P. syringae* pv. *tomato* DC3000(*avrRpt2*) resulted in collapse of the leaf tissue within the first 48 hr after inoculation, which is typical for this incompatible interaction (Figure 1).

*A. brassicicola* is a necrotrophic fungal pathogen that provokes spreading necrotic lesions on leaves. In contrast to basal resistance against *P. syringae*, SA is not required for defense against this pathogen, because Arabidopsis genotypes impaired in SA accumulation retain the strong level of resistance that is characteristic for the wild-type Col-0 plants (Thomma *et al.*, 1998; Van Wees *et al.*, 2003). Basal resistance against *A. brassicicola* is compromised in the phytoalexin-deficient mutant *pad3* and the JA-response mutant *coi1*, indicating that the Arabidopsis phytoalexin camalexin and JA signaling are required for defense against *A. brassicicola* (Thomma *et al.*, 1998; 1999). In our comparative study we used the *pad3* mutant as the susceptible host for studying a compatible Arabidopsis-*A. brassicicola* interaction. After inoculation with *A. brassicicola*, necrotic lesions developed gradually to a size that spanned half the width of the leaf 3 days after inoculation (Figure 1).

Tissue-chewing caterpillars of the cabbage white butterfly (*P. rapae*) are specialists on cruciferous plant species (Van Loon *et al.*, 2000). Defense against caterpillar feeding in plants has been suggested to be mainly regulated by JA-dependent defense responses (Kessler and Baldwin, 2002; Van Poecke and Dicke, 2002). In Arabidopsis, *P. rapae* feeding has been shown to induce expression of JA-responsive genes (Reymond *et al.*, 2000; 2004) and to induce direct and indirect defenses that involve SA, JA, and ET (Reymond *et al.*, 2004; Stotz *et al.*, 2000; 2002; Van Poecke and Dicke, 2004; Van Poecke *et al.*, 2001). Moreover, tomato plants affected in JA production or perception are more susceptible to caterpillar feeding than wild-type plants (Howe *et al.*, 1996; Thaler *et al.*, 2002). In this study, first-instar larvae of *P. rapae* immediately started to feed when they were placed onto the leaf tissue. Caterpillar feeding caused a severe, progressing damage to the leaf tissues (Figure 1).

Western flower thrips (*F. occidentalis*) causes extensive damage on many plant species, including Arabidopsis (Yudin *et al.*, 1986). Thrips are cell-content feeding insects that penetrate single cells with a stylet to suck out the contents (Kindt *et al.*, 2003). JA plays an important role in defense against cell content-feeding herbivores. Tomato mutant *def1*, compromised in JA-signaling, shows enhanced susceptibility to thrips feeding. Moreover, overexpression of JA-inducible prosystemin, a signal peptide involved in the wound-induced



**Figure 1.** Symptom development in *Arabidopsis* upon pathogen and insect attack

Symptom development on *Arabidopsis* leaves at different time points after inoculation/infestation with the necrotizing bacterial leaf pathogen *P. syringae* pv. tomato DC3000(*avrRpt2*), the necrotrophic fungal leaf pathogen *A. brassicicola*, tissue-chewing caterpillars of the cabbage white butterfly (*P. rapae*), cell-content feeding larvae of the Western flower thrips (*F. occidentalis*), or phloem-sucking green peach aphids (*M. persicae*).

expression of protease inhibitors (PIs), resulted in plants highly resistant to thrips damage (Li *et al.*, 2002). *Arabidopsis* leaves infested with *F. occidentalis* displayed white chlorotic spots, so-called silver scars, which were located mainly at the leaf edges. During the course of the experiments, the symptoms became more severe (Figure 1).

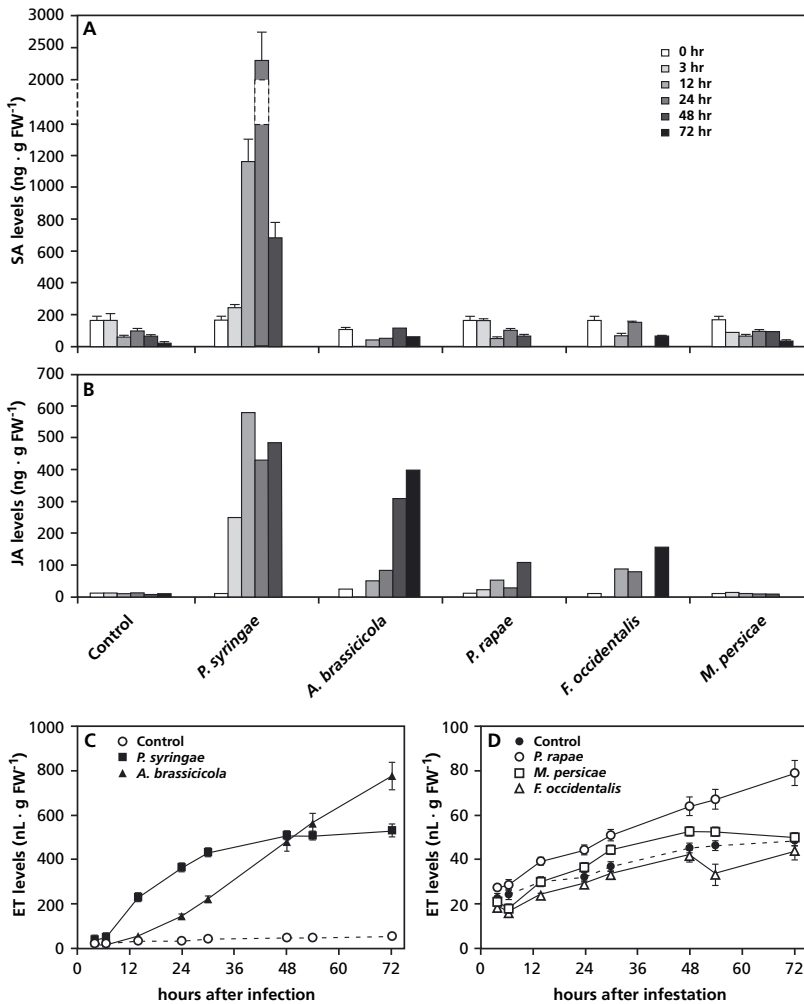
Green peach aphids (*M. persicae*) are generalists that feed on the plant's phloem sap using a sucking mode of action. The aphids carefully maneuver their stylets around the epidermal and mesophyll cells before inserting them into the phloem, thereby inflicting minimal wounding to the plant (Tjallingii and Hogen Esch, 1993). *M. persicae* feeding has been shown to induce the expression of both SA- and JA-responsive genes (Moran and Thompson, 2001), suggesting a role for both signals in defense against aphid feeding. Ellis *et al.*

(2002) demonstrated that *M. persicae* population development is reduced on Arabidopsis mutant *cev1*, which constitutively expresses JA-responsive genes. Moreover, aphid population development was much faster on the JA-insensitive mutant *coi1*, indicating that JA plays an important role in defense against *M. persicae* (Ellis *et al.*, 2002; Moran and Thompson, 2001). In our study, *M. persicae* was allowed to feed for 72 hr. During this 72-hr time course, the aphids fed predominantly on the main vein at the abaxial side of the Arabidopsis leaves without causing any visible symptoms (Figure 1).

### Signal signature

To investigate the dynamics of SA, JA, and ET production during the different Arabidopsis-attacker combinations, we monitored the production of these signals after pathogen and insect attack. Because the progress of disease or damage caused by the pathogens and the insects differed among the Arabidopsis-attacker combinations (Figure 1), the time points for tissue harvest were selected from early to late stages of infection/infestation and, thus, are not always identical for each Arabidopsis-attacker combination. For SA and JA measurements, leaf tissue from 20 plants per plant-attacker combination and untreated controls were harvested at each time point and immediately frozen in liquid nitrogen. For ET determinations, 10 plants per plant-attacker combination were placed in gas-tight vials immediately after pathogen inoculation or insect infestation. Figure 2 shows the production of SA, JA, and ET during the first 72 hr after pathogen or insect attack. *P. syringae* infection induced a strong increase in the production of all three signal molecules. JA production was detectable as early as 3 hr after inoculation, whereas SA and ET levels were increased significantly from 12 hr onwards. Similar to the Arabidopsis-*P. syringae* interaction, inoculation of Arabidopsis with *A. brassicicola* resulted in a strong increase in JA and ET production. Enhanced JA levels were detectable at 3 hr after inoculation, whereas ET levels started to increase between 12 and 24 hr post inoculation. *A. brassicicola* did not induce an increase in SA levels.

None of the insects induced a detectable increase in SA accumulation (Figure 2). Moreover, the magnitude of JA and ET production was much lower in response to insect infestation than during pathogen attack. However, this may be due to the fact that the number of cells contributing to the defense response upon pathogen infection is higher than that upon insect infestation. Feeding by tissue-chewing caterpillars of *P. rapae* induced a modest, but significant increase in ET production, and a clear increase in JA production. Cell content-feeding larvae of the Western flower thrips *F. occidentalis* also induced an increase in JA biosynthesis, whereas ET levels remained unchanged. No changes in the production of JA or ET were detectable in response to infestation of Arabidopsis with phloem-sucking *M. persicae* aphids.



**Figure 2.** Signal signature of Arabidopsis upon pathogen and insect attack

**A.** Endogenous levels of free SA in Arabidopsis plants at different time points after inoculation/infestation with *P. syringae* pv. *tomato* DC3000(*avrRpt2*), *A. brassicicola*, *P. rapae*, *F. occidentalis*, or *M. persicae*. The values presented are means ( $\pm$  SE) of five samples, each consisting of four rosettes that received the same treatment.

**B.** JA levels in Arabidopsis plants at different time points after pathogen inoculation or insect infestation. The values presented are from 20 pooled rosettes that received the same treatment.

**C, D.** Cumulative ET production over a 72-hr period in leaves of Arabidopsis after inoculation with *P. syringae* pv. *tomato* DC3000(*avrRpt2*) or *A. brassicicola* (C), or after infestation with *P. rapae*, *F. occidentalis*, or *M. persicae* (D). The represented values are means ( $\pm$  SE) for 10 plants that received the same treatment.

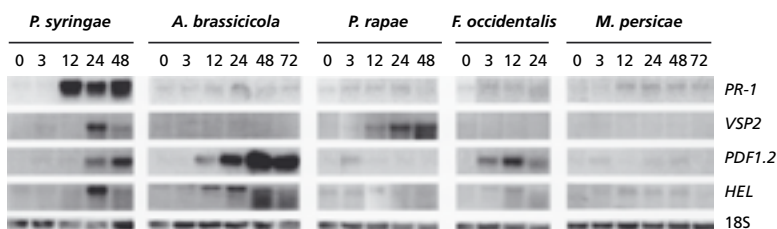
Inoculations with *A. brassicicola* were performed on the Col-0 mutant *pad3-1*, which is a susceptible host for this pathogen. All other inoculations/infestations were carried out with Col-0 plants. Depending on the progress of the symptoms inflicted by the respective pathogens and insects, harvesting of leaf tissue for SA and JA determinations were omitted at some time points (missing bars in A and B). FW, fresh weight.

Together these results demonstrate that the accumulation patterns of SA, JA, and ET differ highly in composition, magnitude, and timing during the different plant-pathogen and plant-insect combinations. The combined patterns of SA, JA, and ET production will subsequently be referred to as the signal signature.

### Attacker-induced marker gene expression

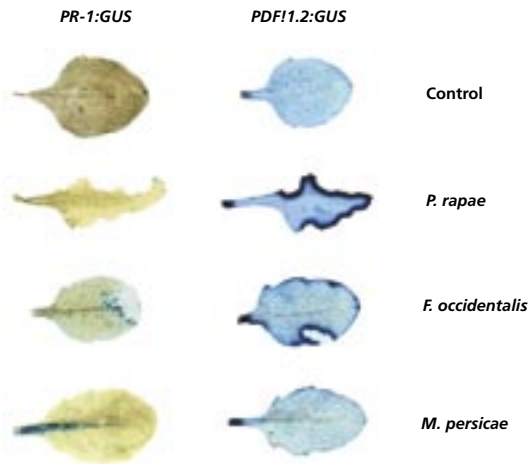
To investigate in how far the specific patterns of defense signal production during each plant-attacker combination correspond with a coordinate activation of SA-, JA-, and/or ET-responsive genes, we first analyzed the expression of the well-characterized marker genes *PR-1* (SA-responsive), *VSP2* (JA-responsive), *PDF1.2* (JA- and ET-responsive), and *HEL* (ET-responsive). To be able to correlate the signal signatures with the gene expression patterns, RNA was isolated from the same leaf samples as those used for the SA and JA determinations. Figure 3 shows that *P. syringae* pv. *tomato* DC3000(*avrRpt2*) induced the expression of all the SA-, JA-, and ET-responsive marker genes, whereas *A. brassicicola* only triggered the JA- and ET-responsive marker genes *PDF1.2* and *HEL*. Furthermore, *P. rapae* and *F. occidentalis* induced the JA-responsive marker genes *VSP2* and *PDF1.2*, respectively. No clear accumulation of any marker gene transcripts could be detected in *M. persicae*-infested plants.

Because aphids damage only a small number of cells while probing for feeding sites, we made use of the transgenic Arabidopsis Col-0 lines *PDF1.2:GUS* and *PR-1:GUS* to examine local aphid-induced marker gene expression in more detail. The *PDF1.2:GUS* and *PR-1:GUS* lines contain a translational fusion of the *uidA* reporter gene with the JA/ET-responsive promoter of the *PDF1.2* gene, and the SA-responsive promoter of the *PR-1* gene, respectively.



**Figure 3.** Northern blot analysis of SA-, JA-, and ET-responsive marker genes in Arabidopsis upon pathogen and insect attack

Transcript levels of SA-responsive (*PR-1*), JA-responsive (*VSP2* and *PDF1.2*), and ET-responsive (*PDF1.2* and *HEL*) marker genes in Arabidopsis leaves at different time points after inoculation/infestation with *P. syringae* pv. *tomato* DC3000(*avrRpt2*), *A. brassicicola*, *P. rapae*, *F. occidentalis*, or *M. persicae*. Equal loading of RNA samples was checked using a probe for 18S rRNA.



**Figure 4.** Histochemical staining of  $\beta$ -glucuronidase (GUS) activity in leaves of transgenic *Arabidopsis PR-1:GUS* and *PDF1.2:GUS* lines after insect feeding

Photographs were taken from representative leaves that were fed on for 24 hr by *P. rapae*, or for 72 hr by *F. occidentalis* or *M. persicae*. Silver scars inflicted by *F. occidentalis* feeding appear as a clear white zone at the edge of the leaf.

No  $\beta$ -glucuronidase (GUS) activity was detected in *PDF1.2:GUS* plants in response to *M. persicae* feeding. In contrast, aphid feeding strongly induced expression of the SA-responsive *PR-1* promoter in the cells surrounding the feeding sites on the main vein (Figure 4).

To similarly investigate local effects of thrips and caterpillar feeding on *PR-1* and *PDF1.2* marker gene expression, GUS activity was also assessed in *F. occidentalis*- and *P. rapae*-infested *PR-1:GUS* and *PDF1.2:GUS* plants. Thrips feeding locally activated the *PR-1* promoter to a moderate level (Figure 4), which was apparently too low to be detected in the RNA isolated from whole rosettes (Figure 3). Damage caused by caterpillar feeding had no effect on GUS activity in *PR-1:GUS* plants. Both *F. occidentalis* and *P. rapae* induced the expression of the *PDF1.2* promoter around the feeding site. The latter was not detected in the RNA from whole rosettes of *P. rapae*-infested plants (Figure 3).

These results indicate that the expression patterns of the marker genes correlate only to a limited extent with the accumulation patterns of the signaling compounds themselves. For instance, JA production in *P. syringae*-infected plants was detectable earlier and to a 5-fold higher level than in *P. rapae*-infested plants. Nevertheless, *VSP2* transcript levels accumulated faster and to a higher level after caterpillar feeding. Furthermore, the timing and magnitude of JA biosynthesis during *P. rapae* and *F. occidentalis* feeding was comparable. However, the expression patterns of JA-responsive genes *PDF1.2* and *VSP2* were clearly different.

**Global expression profiles of *Arabidopsis* upon pathogen and insect attack**

To explore the complexity of the transcriptional changes of *Arabidopsis* in response to pathogen or insect attack, we analyzed the transcriptome of *Arabidopsis* at two time points after pathogen infection or insect infestation using Affymetrix ATH1 whole-genome GeneChips. Because a detailed qualitative analysis of the transcript profiles of each *Arabidopsis*-attacker combination is beyond the scope of this study, we will focus on the comparison of the transcript profiles between the different *Arabidopsis*-attacker combinations. The time points used for the microarray analysis were selected on the basis of the signal signature (Figure 2) and the marker-gene expression (Figure 3), and are listed in Table 1. To be able to relate gene expression to relative SA, JA, and ET levels, RNA was prepared from the same plant material as was used for the determination of the signal signature (Figure 2). RNA was prepared from four biological replicates, each consisting of 5 plants. These replicates were pooled to reduce noise arising from biological variation. The transcript profile of each pool was obtained by hybridization of an Affymetrix ATH1 GeneChip representing approximately 23,750 *Arabidopsis* genes (Redman *et al.*, 2004). After hybridization, expressed genes were identified using GeneChip Operating Software (GCOS), which uses statistical criteria to generate a 'present' or 'absent' call for genes represented by each probe set on the array. The average number of detectable genes (with 'present' call) was 13,729 (60,2%), which is in good agreement with the 60% previously reported by Redman *et al.* (2004).

Expression values from each pooled sample were normalized globally using GCOS. To validate the global normalization, the fold change in expression level of a set of nine genes previously identified as representative, constitutively expressed controls (Kreps *et al.*, 2002), was calculated. As expected, the fold-change ratio in attacker- over mock-treated leaves was close to 1 for most of these genes for all interactions and time points tested (Table 1).

To identify attacker-responsive genes, the transcript profile of each selected time point of each *Arabidopsis*-attacker combination was compared to the transcript profile of their respective mock-treated control plants that were grown under identical conditions and were harvested at the same two time points as the attacker-induced plants. To identify a robust set of pathogen- and insect-responsive genes, we chose an experimental set-up in which we selected for genes of which changes in expression level were evident during the whole time-frame monitored for each of the *Arabidopsis*-attacker combinations. The following conservative selection criteria were applied. First, per *Arabidopsis*-attacker combination the expression level had to be detectable (*P*-flag generated by GCOS) and the hybridization intensity had to be >40 units in at least two out of four data sets. Second, the change in expression level in attacker-treated

**Table 1.** Fold-change ratio of representative constitutively expressed control genes in the different Arabidopsis-attacker combinations compared to mock-treated Arabidopsis plants

Annotation	AGI No.	Fold-change <sup>1</sup>											
		<i>P. syringae</i>		<i>A. brassicic.</i>		<i>P. rapae</i>		<i>F. occident.</i>		<i>M. persicae</i>			
		t=12	t=24	t=24	t=48	t=12	t=24	t=12	t=24	t=48	t=72	AVG ± SD	
Polyubiquitin, UBQ10	At4g05320	1.06	0.91	0.74	0.83	1.06	0.75	1.02	1.05	1.35	1.67	1.04 ± 0.28	
Eucaryotic init. Fact. eIF-4A1	At3g13920	1.06	1.36	1.07	1.25	0.88	0.81	0.86	1.00	0.87	1.18	1.03 ± 0.19	
Aquaporin, PIP-1B	At2g45960	0.36	0.75	1.43	1.08	0.96	1.07	0.85	0.92	0.48	0.22	0.81 ± 0.37	
40S ribosomal protein S16	At2g09990	0.80	1.09	1.10	1.09	0.79	0.95	0.89	1.04	0.75	1.01	0.95 ± 0.14	
Actin 2	At3g18780	0.65	0.56	1.23	0.86	0.85	0.77	0.79	0.87	0.84	1.55	0.90 ± 0.29	
Pl. membr. H <sup>+</sup> -ATPase, AHA1	At2g18960	0.74	1.58	1.03	0.86	1.18	1.13	0.89	1.11	1.15	1.11	1.08 ± 0.23	
Tubulin, β-4	At5g44340	0.85	0.62	0.88	0.99	0.88	0.79	0.82	0.75	1.20	1.83	0.96 ± 0.34	
Calmodulin-1	At5g37780	1.81	0.79	0.75	1.02	1.02	0.81	1.10	1.02	2.28	3.16	1.38 ± 0.79	
Ca-dep. protein kinase, CPK3	At4g23650	1.10	1.27	0.98	1.36	0.91	0.87	0.95	0.95	1.37	1.22	1.10 ± 0.19	
AVG		0.94	0.99	1.02	1.04	0.95	0.88	0.91	0.97	1.14	1.44		
±		±	±	±	±	±	±	±	±	±	±		
SD		0.40	0.35	0.22	0.18	0.12	0.14	0.10	0.11	0.52	0.80		

<sup>1</sup> Fold-change ratios (attacker/mock) are based on gene expression profiles of leaves of Col-0 plants at indicated time points after inoculation/infestation. AVG, average; SD, standard deviation

**Table 2.** Comparison of microarray and northern blot data of the marker genes *PR-1*, *PDF1.2*, and *HEL* in different Arabidopsis-attacker combinations.

Attacker		Fold-change					
		<i>PR-1</i>		<i>PDF1.2</i>		<i>HEL</i>	
		Mic <sup>1</sup>	Nor <sup>2</sup>	Mic	Nor	Mic	Nor
<i>P. syringae</i>	12 hr	60.4	50.5	-1.7	1.1	1.7	1.1
	24 hr	38.3	25.5	7.7	15.7	2.7	8.8
<i>A. brassicicola</i>	24 hr	-1.5	<1	5.0	50.2	2.7	1.4
	48 hr	2.5	1.4	126.7	77.5	12.5	2.2
<i>P. rapae</i>	12 hr	-1.2	<1	-1.4	<1	-2.2	<1
	24 hr	3.1	1.4	-1.4	1.4	-1.1	<1
<i>F. occidentalis</i>	12 hr	-1.2	1.2	6.6	6.3	2.8	1.5
	24 hr	2.6	1.3	11.4	27.0	3.3	3.2
<i>M. persicae</i>	48 hr	2.8	1.7	12.4	<1	4.0	2.1
	72 hr	5.1	1.3	-2.2	<1	4.0	1.3

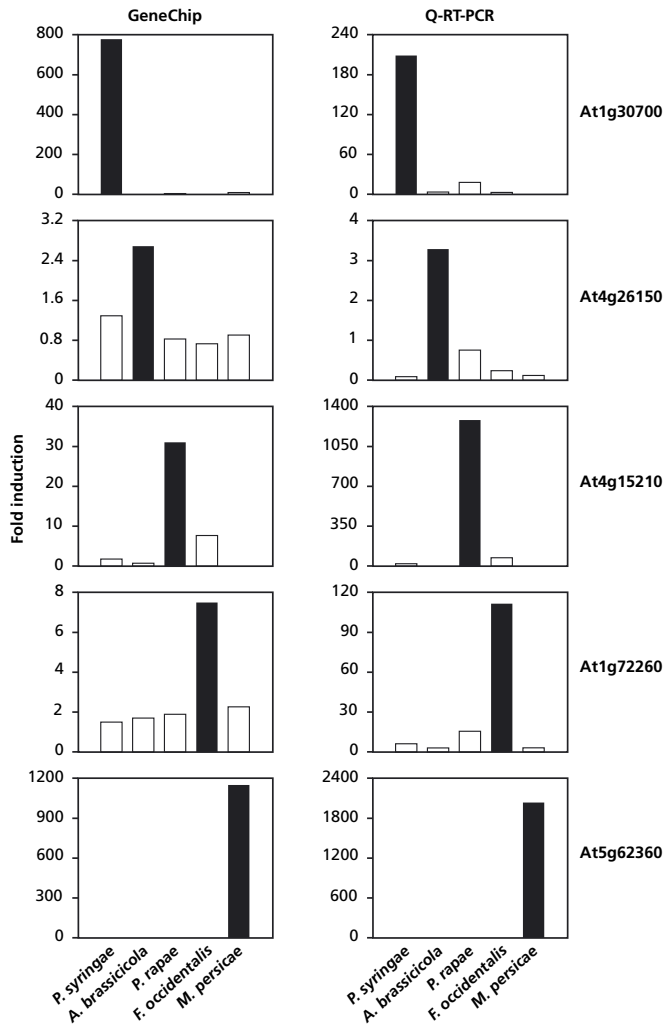
<sup>1</sup> Fold changes are marked in **dark red** when the corresponding genes were selected as responsive to the attacker indicated (consistently showed a >2-fold change in the same direction (up or down) in the microarray data sets (Mic)). Fold-changes are marked in **dark blue** when the corresponding genes did not consistently show a >2-fold change in the same direction (up or down) in the microarray data sets.

<sup>2</sup> Signal intensities on the northern blots were quantified using a Phosphor Imager and compared to the untreated control. The calculated fold-changes are given in the same type as the corresponding fold-changes in the microarray analysis when they were comparable. **Red** and **blue** are given when the fold-change on the northern blot was in the same direction but, in contrast to the microarray analysis, below 2-fold.

leaves compared to that in mock-treated control leaves had to be at least 2-fold. To avoid false positives we required the changes to occur at both time points and to be in the same direction. Only those probe sets were selected that met these stringent selection criteria at both time points tested. The attacker-induced genes corresponding to the selected probe sets are listed in Supplements 1 to 5.

### Validation of microarray data

To validate the GeneChip results we compared the relative expression values of the marker genes *PR-1*, *PDF1.2*, and *HEL* with the relative mRNA levels on the northern blots. *VSP2* was left out of this analysis because it is not represented on the ATH1 GeneChip. Hybridization signals on the northern blots were quantified using a Phosphor Imager and the fold-change relative to the respective controls calculated. Table 2 shows that out of 30 combinations tested (3 marker genes x 5 Arabidopsis-attacker combinations x 2 time points) 29 matched with the microarray data, indicating that the relative



**Figure 5.** Comparison of microarray and Q-RT-PCR analysis of five attacker-specific genes in the different Arabidopsis-attacker combinations

Fold induction of five attacker-specific genes (At1g30700, At4g26150, At4g15210, At1g72260, and At5g62360) after infection/infestation of Arabidopsis by *P. syringae*, *A. brassicicola*, *P. rapae*, *F. occidentalis*, or *M. persicae*. On the left, the fold-change patterns from the microarray analysis. On the right, the fold-change patterns from the Q-RT-PCR analysis.

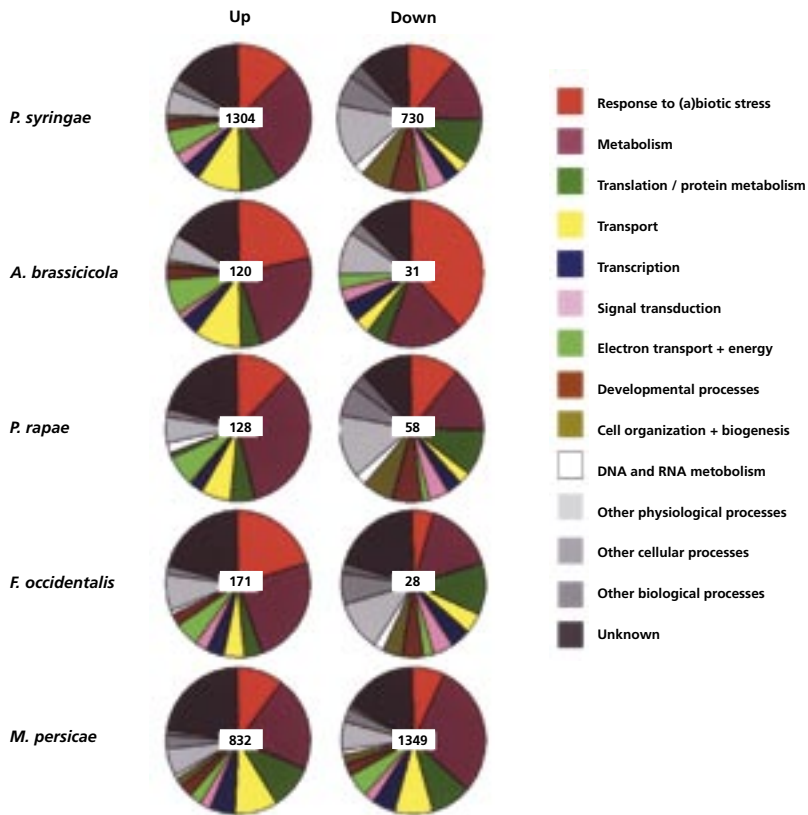
expression levels of the marker genes correlated well between GeneChip and northern blot hybridization. In addition, we determined the transcript levels of five attacker-specific genes (At1g30700, At4g26150, At4g15210, At1g72260, At5g62360) in each of the five Arabidopsis-attacker combinations and their respective mock-treated controls, using quantitative real-time PCR (Q-RT-PCR). Figure 5 shows the fold-change induction of the selected genes in

the different Arabidopsis-attacker combinations as determined by microarray analysis (left panel) and Q-RT-PCR (right panel). Although fold induction in gene expression, especially for low abundant mRNAs, has been shown to differ between the two methods (Czechowski *et al.*, 2004), the relative expression patterns of the five attacker-specific genes were highly similar, indicating that the relative expression levels of the genes tested correlated well between GeneChip and Q-RT-PCR analysis.

To further validate the GeneChip data obtained, we compared the selected pathogen- and insect-responsive genes with those identified in other transcript profiling studies in which the same or similar Arabidopsis-attacker combinations were used (Glazebrook *et al.*, 2003; Moran *et al.*, 2002; Reymond *et al.*, 2004; Reymond *et al.*, 2000; Tao *et al.*, 2003; Van Wees *et al.*, 2003; Verhagen *et al.*, 2004). Although the experimental set-up, such as age of the plant material upon harvest, time points after inoculation and the type of microarray used, often differed in these studies, a large number of genes behaved similarly (data not shown). For instance, 65% of all the *P. syringae*-responsive genes identified in our study (Supplement 1) that are also represented on the Arabidopsis Genome 8K array of Affymetrix, were also identified as being *P. syringae*-responsive by Tao *et al.* (2003). Moreover, 79% of all the *A. brassicicola*-responsive genes identified in our study that are also present on the Arabidopsis Genome 8K array, were also identified as being *A. brassicicola*-responsive by Van Wees *et al.* (2003). All together, these results indicate that our experimental set-up and stringent selection criteria resulted in the selection of a robust set of pathogen- and insect-responsive genes.

### Functional analysis of differentially expressed genes

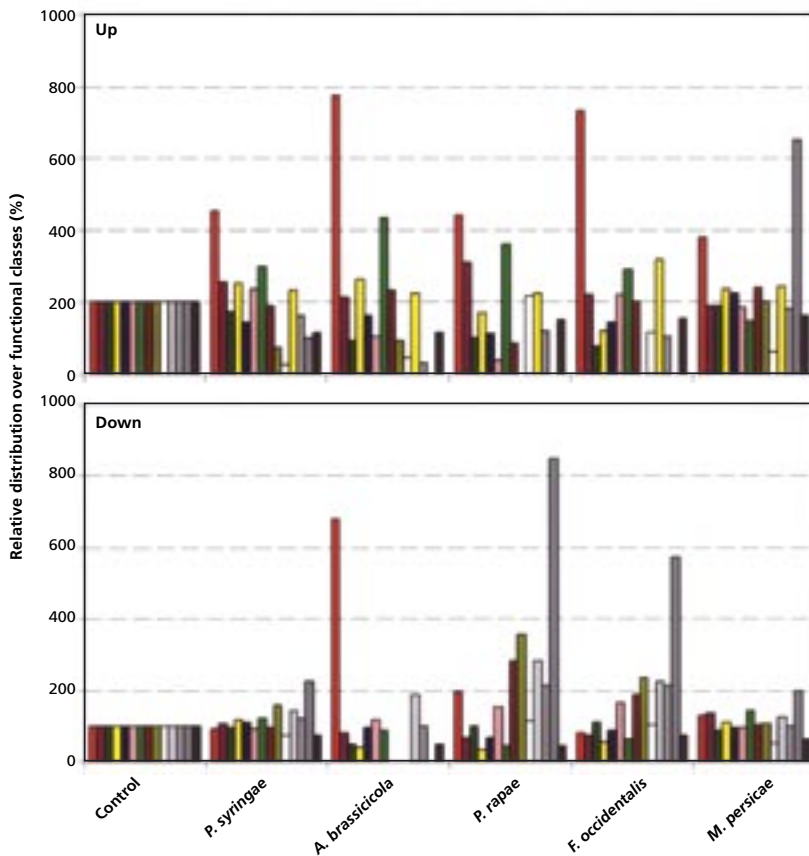
All differentially expressed genes identified in the five Arabidopsis-attacker combinations were classified according to their functional categories derived from the Gene Ontology tool at The Arabidopsis Information Resource (TAIR) (<http://www.Arabidopsis.org>) (Rhee *et al.*, 2003). The distribution of the identified probe sets over the different functional categories is shown in Figure 6. To evaluate the importance of a given functional category, the percentage of differentially expressed genes belonging to each functional category was compared to the degree of representation of the respective functional category in the genome. Figure 7 shows the results of this comparison for the up- and down-regulated genes. The predominant functional category that is overrepresented in the up-regulated gene sets of four out of five Arabidopsis-attacker combinations represent genes involved in the response to abiotic and biotic stress. In the Arabidopsis-*M. persicae* interaction, genes from this category are overrepresented as well, although the predominant overrepresented category represents genes involved in so-far unspecified biological processes (“other biological processes”).



**Figure 6.** Functional analysis of differentially expressed gene sets

Distribution of the differentially expressed genes identified in the Arabidopsis-attacker combinations over the functional categories. The number of up- or down-regulated genes is given in the center of the respective pies. Classification in functional categories was performed essentially according to the Gene Ontology tool of TAIR. Genes belonging to the functional category 'response to abiotic and biotic factors' and 'response to stress' were grouped in a single functional category designated 'response to abiotic and biotic stress'.

Of the differentially expressed genes that are down-regulated during the Arabidopsis-*A. brassicicola* interaction, genes involved in the response to abiotic and biotic stress are clearly overrepresented. This indicates that, besides differential activation, also repression of stress-related genes occurs during the response of Arabidopsis to this pathogen. In the Arabidopsis-*P. rapae* and Arabidopsis-*F. occidentalis* interactions, genes involved in so-far unspecified biological processes ("other biological processes") are clearly overrepresented in the down-regulated gene sets. However, the specific biological gene functions are diverse, impeding any speculation as to their biological relevance. In the interactions of Arabidopsis with *P. syringae* and *M. persicae* none of the



**Figure 7.** Functional analysis of differentially expressed gene sets

Degree of overrepresentation of the differentially expressed genes in the functional categories. The distribution of the differentially expressed genes over the functional categories is presented relative to the distribution of all genes on the Affymetrix ATH1 array (set at 100% for each functional category).

functional categories are clearly overrepresented among the down-regulated genes.

### Comparison of transcriptome changes induced by pathogen and insect attack

Table 3 shows the number of genes that are consistently up- or down-regulated in the different Arabidopsis-attacker combinations (complete lists in Supplements 1 to 5). Of all the attackers investigated, *M. persicae* induced the largest number of changes (2181). This is remarkable because aphid feeding caused virtually no visual symptoms in comparison to the extensive damage caused by the other attackers. *P. syringae* infection resulted in a similar number

**Table 3.** Analysis of probe sets showing a consistent 2-fold change in time in *Arabidopsis* leaves upon infection/infestation with *P. syringae*, *A. brassicicola*, *P. rapae*, *F. occidentalis*, or *M. persicae*.

Attacker	Signal signature <sup>1</sup>			Consistent changes <sup>2</sup>			Overlap (%) <sup>3</sup>				
	SA	JA	ET	up	down	total	<i>P.s.</i>	<i>A.b.</i>	<i>P.r.</i>	<i>F.o.</i>	<i>M.p.</i>
<i>P. syringae</i>	+++	+++	+++	1304	730	2034	100	5	5	7	12
<i>A. brassicicola</i>	-	+++	+++	120	31	151	68	100	5	22	13
<i>P. rapae</i>	-	++	+	128	58	186	52	4	100	39	7
<i>F. occidentalis</i>	-	++	-	171	28	199	72	17	36	100	18
<i>M. persicae</i>	-	-	-	832	1349	2181	12	1	1	2	100

<sup>1</sup> Relative amounts of signal molecules produced in *Arabidopsis* in response to pathogen or insect attack as judged from Figure 2. +++ = high levels; ++ = moderate levels; + = low levels; - no change compared to control.

<sup>2</sup> Number of probe sets representing attacker-responsive genes with a consistent >2-fold change over time in the same direction (up or down) (complete lists in Supplement 1 to 5).

<sup>3</sup> Pair-wise comparison of the percentage of overlap between probe sets. Percentages are presented relative to the total number of changes induced by the attacker given in the same row (e.g. 68% of all *A. brassicicola*-induced changes are also induced by *P. syringae*). *P.s.*, *P. syringae*; *A.b.*, *A. brassicicola*; *P.r.*, *P. rapae*; *F.o.*, *F. occidentalis*; *M.p.*, *M. persicae*.

of consistent changes (2034), whereas the number of consistent changes in the other *Arabidopsis*-attacker combinations was much lower (151 to 199). It must be noted that in all *Arabidopsis*-attacker combinations, many more genes showed a more than 2-fold change in expression at a single point in time. Because these changes are not as robust as the consistent changes, they were not analyzed further.

To evaluate the complexity of the transcriptional changes induced during the five different *Arabidopsis*-attacker combinations, we made a pair-wise comparison of the overlap between the selected probe sets. Table 3 shows that in the majority of the comparisons the overlap is relatively small, indicating that most of the differentially expressed genes are specific for the respective *Arabidopsis*-attacker combinations. However, more than 50% of all consistent changes elicited by *A. brassicicola* (68%), *P. rapae* (52%), and *F. occidentalis* (72%), are also consistently triggered by *P. syringae*, suggesting that these genes are commonly activated/repressed during these *Arabidopsis*-attacker interactions. Interestingly, these four attackers all induced a considerable increase in JA levels (Figure 2), suggesting that JA may be the common regulator of the overlapping gene sets.

To investigate the role of JA in the regulation of the overlapping gene sets, we identified probe sets representing JA-responsive genes among the selected attacker-responsive genes. To this end, 5-week-old Col-0 plants were treated with 0.05 mM MeJA and harvested 0, 1, 3, and 6 hr later. RNA from these

**Table 4.** Overlap of JA-responsive genes showing a consistent 2-fold change in time in Arabidopsis leaves after infection/infestation with JA-inducing attackers *P. syringae*, *A. brassicicola*, *P. rapae*, or *F. occidentalis*.

Attacker	Consistent changes <sup>1</sup>		% Overlap of JA-responsive genes <sup>2</sup>			
	total	JA-responsive	<i>P.s.</i>	<i>A.b.</i>	<i>P.r.</i>	<i>F.o.</i>
<i>P. syringae</i>	2034	652 (32%)	100	9	9	17
<i>A. brassicicola</i>	151	67 (44%)	85	100	9	34
<i>P. rapae</i>	186	103 (55%)	66	6	100	54
<i>F. occidentalis</i>	199	138 (69%)	80	17	41	100

<sup>1</sup> Total number of probe sets with consistent >2-fold change (up or down) over time in response to the attacker indicated in the same row (from Table 3), and the number of genes from the "total" list that showed a >2-fold change in the same direction in response to treatment with 0.05 mM MeJA. The percentage of these JA-responsive genes is given between parentheses.

<sup>2</sup> Percentage overlap between the JA-responsive genes among the selected attacker-induced probe sets. Percentages are given relative to the total number of JA-responsive genes induced by the attacker given in the same row (e.g. 85% of all JA-responsive, *A. brassicicola*-induced changes are also *P. syringae*-responsive). *P.s.*, *P. syringae*; *A.b.*, *A. brassicicola*; *P.r.*, *P. rapae*; *F.o.*, *F. occidentalis*.

plants was used to prepare probes for the hybridization of Affymetrix ATH1 GeneChips. Probe sets showing a >2-fold change (up or down) on at least two of the time points tested were selected as described above. The resulting 2,209 probe sets were considered to represent JA-responsive genes (Supplement 6). Comparison of these JA-responsive genes among the selected attacker-responsive probe sets revealed that 32% of the *P. syringae*-responsive genes are responsive to MeJA (Table 4). The percentages of JA-responsive genes among the *A. brassicicola*-, *P. rapae*-, and *F. occidentalis*-induced changes were even higher (44%, 55%, and 69%, respectively), indicating that JA plays a dominant role in the transcriptional reprogramming of Arabidopsis in response to these attackers. Pair-wise comparisons of the overlap between JA-responsive genes in the four Arabidopsis-attacker combinations, revealed that of all JA-responsive, *P. rapae*-induced changes, 66% is also induced by *P. syringae* (Table 4). In the Arabidopsis-*F. occidentalis* and the Arabidopsis-*A. brassicicola* interactions, this percentage is even higher (80% and 85%, respectively), indicating that the JA-induced defense responses triggered by these attackers show considerable overlap. However, this does not hold for all Arabidopsis-attacker combinations. For instance, when the JA-responsive genes among the *P. rapae*- and *F. occidentalis*-induced changes were compared with the JA-responsive genes among the *A. brassicicola*-induced ones, the overlap was relatively low (6 to 17%). These results indicate that although attackers with very different modes of action (e.g. *F. occidentalis* and *P. syringae*) may induce similar sets of JA-responsive genes, the majority of the JA-responsive genes are affected in an

attacker-specific manner, indicating that other factors besides JA shape the final outcome of the defense response.

## Discussion

Plants require a broad range of defense mechanisms to effectively combat invasion by microbial pathogens or attack by herbivorous insects. These mechanisms include pre-existing physical and chemical barriers, as well as inducible defense responses that become activated upon pathogen infection or insect herbivory. A concerted action of these defensive activities helps the plant to minimize damage caused by the attacker. The signal molecules SA, JA, and ET have been implicated in many plant-pathogen and plant-insect interactions (Dicke and Hilker, 2003; Pieterse and Van Loon, 1999). Despite the evident overlap in signaling that is triggered upon pathogen or insect attack, the plant response is highly dependent on the plant-attacker combination. Little is known about how plants co-ordinate attacker-induced signals into specific defense responses. A well-accepted hypothesis is that modulation of the different defense signaling pathways involved plays an important role in this process (Reymond and Farmer, 1998). Although ample information is available on the role of SA, JA, and ET in the response of plants to certain pathogens and insects, the information is often highly specific for a given plant-pathogen or plant-insect interaction. Moreover, the different studies are often characterized by unique experimental conditions. Here, we attempted to gain insight into the dynamics of the response of a single plant species (*Arabidopsis thaliana*) to a variety of microbial and herbivorous attackers under identical conditions. This approach allowed us to compare the dynamics of signal production and the transcriptional reprogramming of *Arabidopsis* upon attack by pathogens and insects with very different modes of attack.

### **Correlation between signal signature and marker gene expression**

Gene expression profiles and SA, JA, and ET production were examined simultaneously during the entire period between inoculation/infestation and the occurrence of the resulting severe symptoms or damage (Figure 1). Because aphids did not cause any visible symptoms, the response of *Arabidopsis* to this attacker was monitored over a 72-hr time course. All other attackers caused a significant increase in the production of one or more of the signals tested (Figure 2). The accumulation patterns of SA, JA, and ET during the different *Arabidopsis*-attacker interactions clearly differed in composition, magnitude, and timing. This so-called signal signature was reflected in the expression patterns of

the well-characterized marker genes *PR-1*, *VSP2*, *PDF1.2*, and *HEL* (Figure 3). For instance, *P. syringae* infection caused a considerable increase in SA, JA, and ET production, and was associated with the subsequent activation of all the SA-, JA-, and ET-responsive marker genes tested. Furthermore, *A. brassicicola* infection caused a significant increase in both JA and ET levels, resulting in the activation of the JA- and ET-responsive marker genes *PDF1.2* and *HEL*. However, in some Arabidopsis-attacker combinations the signal signature correlated only to a limited extent with the expression patterns of the marker genes. The high levels of JA produced by Arabidopsis in response to infection by *A. brassicicola* resulted in the activation of the JA-responsive gene *PDF1.2*, but not in that of the JA-responsive gene *VSP2*. Moreover, although *P. rapae* and *F. occidentalis* induced comparable levels of JA in Arabidopsis, *VSP2* was activated in the Arabidopsis-*P. rapae* interaction, whereas *PDF1.2* was not. Conversely, *F. occidentalis* triggered the expression of *PDF1.2* but not that of *VSP2*. Hence, it must be concluded that the signal signature of a given plant-attacker combination plays a primary role in the orchestration of the plant's defense response, but additional layers of regulation lead to differential marker gene expression.

#### **Attacker-induced transcriptional changes**

The goal of the microarray analysis was to explore the complexity of the transcriptional reprogramming initiated by the different pathogens and insects in relation to the observed Arabidopsis-attacker signal signatures, and to identify robust sets of attacker-responsive genes. To this end, we applied stringent selection criteria to identify genes that show a consistent change in expression during pathogenesis and herbivore feeding. Depending on the Arabidopsis-attacker combination, 151 to 2181 genes showed a consistent change in expression over time. Surprisingly, aphid feeding triggered the largest number of consistent changes in gene expression, even though these insects caused the least symptoms of all attackers tested and did not induce detectable changes in SA, JA, and ET levels (Figures 1 and 2). In contrast to the other four Arabidopsis-attacker combinations, a large proportion of the differentially expressed genes in the Arabidopsis-aphid interaction was down-regulated (62% versus 14–36% in the other combinations). A relatively large fraction of the down-regulated genes is involved in plant metabolism, confirming previous findings that demonstrate that aphids are major manipulators of plant physiology and nutrition status (Davies *et al.*, 2004). Previously, Moran and co-workers (Moran *et al.*, 2002; Moran and Thompson, 2001) identified 19 *M. persicae*-responsive genes in Arabidopsis by northern blot and small-scale microarray analysis. Thirteen of these genes (68%) were among the 2181 identified as being consistently responsive to *M. persicae* in our GeneChip

analysis, including the SA-responsive genes *PR-1* (At2g14160) and *PR-2* ( $\beta$ -1,3-glucanase; At3g57260). Although *PR-1* transcript levels were barely detectable on the northern blots (Figure 3), they were clearly expressed in the cells surrounding the feeding sites on the main veins of the *PR-1:GUS* reporter line (Figure 4). These results indicate that significant local changes in gene expression can be identified by microarray analysis, while escaping from identification by northern blot analysis.

A large proportion of the gene sets identified in our study as being attacker-responsive, has also been identified in comparable studies (Glazebrook *et al.*, 2003; Reymond *et al.*, 2000; Tao *et al.*, 2003; Van Wees *et al.*, 2003; Verhagen *et al.*, 2004). For instance, Reymond *et al.* (2000) identified 17 genes showing a >2-fold increase in expression level in response to *P. rapae* feeding using a small dedicated microarray with probes for 150 Arabidopsis genes. Of the genes also represented on the ATH1 chip, 59% showed a consistent >2-fold increase in our Arabidopsis-*P. rapae* data sets, even though different time points after infestation (3 hr in the study by Reymond *et al.* versus 12 and 24 hr in our study) and different larval stages (L4/L5 in the study of Reymond *et al.* versus L1/L2 in our study) were tested. Furthermore, 65% of the *P. syringae*-responsive genes that were identified in our study (and were present on both the ATH1 and the Affymetrix 8k array), were also identified by Tao *et al.* (2003). Similarly, 79% of the *A. brassicicola*-responsive genes were also identified by Van Wees *et al.* (2003), whom also used the susceptible phytoalexin-deficient mutant *pad3* to study the Arabidopsis-*A. brassicicola* interaction. Together, these data indicate that the gene sets that were selected in this study are to a large extent representative for the different Arabidopsis-attacker combinations used. It must, however, be noted that to achieve a maximal response of Arabidopsis to *P. syringae* infection, we made use of an avirulent strain of the pathogen. Although it has been suggested that the difference in the transcriptional response of Arabidopsis to virulent and avirulent strains of *P. syringae* is predominantly quantitative (Tao *et al.*, 2003), it can not be excluded that a small proportion of the selected genes are specific for the incompatible interaction.

Genes showing a >2-fold change at a single time point are either part of a transient response or false positives and, thus, are unlikely to be identified consistently when bioassays are performed under different experimental conditions. Although some of these genes may play an important role in the response of Arabidopsis to the attacker involved, the scope of this study was not to provide a qualitative in depth analysis of individual gene sets that are differentially expressed in the different Arabidopsis-attacker combinations, but to explore the complexity of the transcriptional changes in the response of Arabidopsis to attack by different pathogens and insects. Therefore, we limited our analysis to those genes that showed a robust change in expression and

disregarded all others. The selected robust gene sets obtained with the whole-genome ATH1 arrays can be related to actual SA, JA, and ET levels and will be of value for more detailed analyses of individual Arabidopsis-attacker interactions. The data sets of all ATH1 array hybridisations used in this study are available via [The Arabidopsis Information Resource (TAIR): [www.Arabidopsis.org](http://www.Arabidopsis.org) Nottingham Arabidopsis Stock Centre International Affymetrix Service (NASCarry: [affymetrix.Arabidopsis.info](http://affymetrix.Arabidopsis.info)].

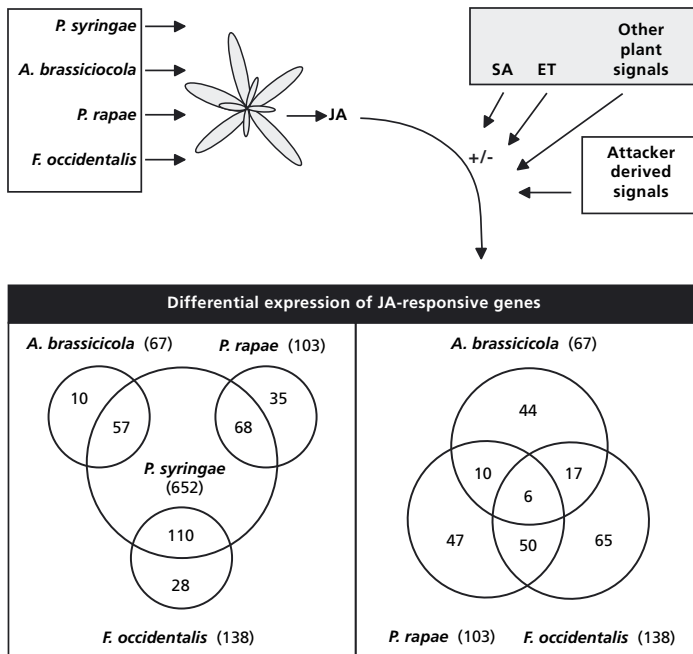
### **Stress-related genes are overrepresented in all Arabidopsis-attacker combinations**

To gain insight into the function of the differentially expressed genes, we categorized their biological function essentially according to the Gene Ontology tool of TAIR. Some of these functional categories cover a relatively large proportion of the Arabidopsis genome, e.g. genes in the functional category ‘metabolism’ represent 21.7% of all annotated genes, while genes in the category ‘response to abiotic and biotic stress’ represent only 5.6% of the genome. Thus, information on the percentage of selected genes in a given functional category is biased by the degree of representation of this category in the genome. To identify functional categories in which a relatively large proportion of the genes show a consistent change in expression in response to pathogen or insect attack, we compared the number of identified genes in a given functional category with the degree of representation of this category in the whole genome. In this way, functional categories that are overrepresented in the selected differentially expressed genes sets were readily identified (Figure 7). In all Arabidopsis-attacker combinations tested, the number of up-regulated genes predicted to be involved in the response to biotic and abiotic stress was 2- to 4-fold higher than expected on the basis of representation of this category in the genome. Evidently, differential expression of a large proportion of genes from this category plays an important role in the response of Arabidopsis to pathogen and insect attack. However, when looking at the absolute percentages of representation of the genes in the different functional categories (Figure 6), the contribution of stress-related genes in the investigated interactions is not immediately clear. For instance, of all consistently up-regulated genes in the different Arabidopsis-attacker combinations 10.6 to 21.7% belongs to the functional category ‘response to abiotic and biotic stress’, while a considerably larger proportion of the genes (20.8 to 33.8%) fall into the functional category ‘metabolism’ (Figure 5A). Thus, assessment of the distribution of the identified gene sets over the different functional classes as a function of the degree of representation of these functional categories in the genome, makes it possible to better weigh the importance of a given functional category in the plant response studied.

### Complexity of transcriptional reprogramming upon pathogen and insect attack

To explore the complexity of transcriptional changes induced by the different *Arabidopsis* attackers used, we compared the overlap between gene sets. Because both *P. syringae* and *M. persicae* induced, by far, the largest number of consistent changes (10- to 14-fold more genes than *A. brassicicola*, *P. rapae*, and *F. occidentalis*), it is evident that the transcriptional response of *Arabidopsis* to these very different attackers is highly complex. In the case of *P. syringae*, this may be related to the fact that infection of *Arabidopsis* by this pathogen results in the production of high levels of SA, JA, and ET, each of which may activate different sets of genes. In the case of *M. persicae* feeding, however, none of these signals tested was detectable. Evidently, the onset of the large transcriptional reprogramming elicited by these phloem-feeding insects is not based on the production of high overall levels of SA, JA, or ET, suggesting that the responses of *Arabidopsis* to *P. syringae* and *M. persicae* is highly unrelated. Indeed, most of the transcriptional changes induced by *P. syringae* or *M. persicae* were unique. Nonetheless, 253 genes (141 up-regulated genes and 112 down-regulated genes; data not shown) of all consistently induced changes in the *Arabidopsis*-*P. syringae* and the *Arabidopsis*-*M. persicae* interaction overlapped. Thus, although both attackers have very different modes of action and trigger a highly dissimilar signal signature, a large number of *Arabidopsis* genes are recruited in response to both attackers. However, these overlapping genes only represent 12% of the total number of consistent changes identified in both interactions and, thus, may only contribute to a limited extent to the overall defense reaction.

Compared to *P. syringae* and *M. persicae*, *A. brassicicola*, *P. rapae*, and *F. occidentalis* induced only a relatively low number of consistent changes in gene expression (151 to 199 up- or down-regulated genes). A small number of these genes (6) showed a consistent change in all three *Arabidopsis*-attacker combinations (data not shown). Pair-wise comparison of the differentially expressed gene sets revealed an overlap of 4% (*P. rapae* versus *A. brassicicola*), 17% (*F. occidentalis* versus *A. brassicicola*), and 39% (*P. rapae* versus *F. occidentalis*). In these three *Arabidopsis*-attacker interactions, JA is a dominant component of the signal signature produced. Indeed, 44 to 69% of all differentially expressed genes identified in these three *Arabidopsis*-attacker combinations were also found to be responsive to exogenous application of MeJA (Table 4), indicating that JA-responsive gene expression plays a central role in the response of *Arabidopsis* to infection/infestation by all three attackers. However, the majority (94 to 46%) of these MeJA-responsive genes showed an attacker-specific expression pattern in pair-wise comparisons between the differentially expressed gene sets. This may be partly explained by differences in sampling time points, but on all time



**Figure 8.** Differential expression of JA-responsive genes upon attack by JA-inducing pathogens and insects

Attack of Arabidopsis by *P. syringae*, *A. brassicicola*, *P. rapae*, or *F. occidentalis*, results in a strong increase in the production of JA, and a concomitant change in the expression of a large number of JA-responsive genes (numbers are given between parenthesis). Nevertheless, the overlap among the JA-responsive genes between the different Arabidopsis-attacker combinations is relatively low (number of overlapping genes between the indicated Arabidopsis-attacker combinations are given in the Venn diagrams). SA and ET have been demonstrated to cross-communicate with the JA pathway. Hence, depending on the amount and timing of their production, SA and ET may have positive or negative effects on the expression of specific sets of JA-responsive genes. In addition, so far unidentified plant- or attacker-derived signals, or physiological conditions that are inflicted by the attacker, may be involved in modulating JA-responsive gene expression.

points tested JA levels were clearly elevated. Hence, the sets of JA-responsive genes that are differentially activated or repressed in the different Arabidopsis-attacker combinations are highly divergent, suggesting that so far unidentified regulatory processes play an important role in modulating the final outcome of the defense response. Figure 8 shows a model of how invasion by JA-inducing attackers may result in the activation of differential sets of JA-responsive genes. Similar models can be drawn for genes that are regulated by other defense-related signals such as SA and ET, resulting in a network of interconnecting signaling pathways that provides the plant with a powerful regulatory potential to fine-tune its defense response.

In conclusion, we demonstrated that Arabidopsis is highly adapted in its response to pathogens and herbivorous insects with very different modes

of attack. Depending on the *Arabidopsis*-attacker combination, the signal molecules SA, JA, and ET are produced with large differences in both quantity and timing. We identified differentially expressed gene sets that over time show a consistent change in expression for each of the *Arabidopsis*-attacker combinations. In all cases, stress-related genes are clearly overrepresented in the gene sets identified. In four of the five *Arabidopsis*-attacker combinations tested, JA plays an important role in the differential regulation of a large proportion of the activated/repressed genes. Nevertheless, the vast majority of the JA-responsive changes are specific for each plant-attacker combination. Evidently, signal molecules such as JA play an important role in the primary response of the plant to pathogen and insect attack. However, additional layers of regulation obviously shape the outcome of the defense reaction. Pathway cross-talk or effects of so far unidentified regulatory factors may play an important role in the fine-tuning of the plant's response to pathogens and insects. The nature and importance of these regulatory processes will be a challenging topic for future research.

## Materials and methods

### Cultivation of plants

Seeds of *Arabidopsis thaliana* accession Col-0 and the phytoalexin-deficient Col-0 mutant *pad3-1* (Glazebrook and Ausubel, 1994) were sown in quartz sand. Two-week-old seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min. Plants were cultivated in a growth chamber with a 8-h day ( $200 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  at  $24^\circ\text{C}$ ) and 16-hr night ( $20^\circ\text{C}$ ) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing  $10 \mu\text{M}$  Sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

### Pathogen bioassays

Inoculations with the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 was performed as described previously (Van Wees *et al.*, 1999). Briefly, *P. syringae* pv. *tomato* DC3000 with the plasmid pV288 carrying avirulence gene *avrRpt2* (Kunkel *et al.*, 1993) was cultured overnight at  $28^\circ\text{C}$  in liquid King's medium B (King *et al.*, 1954), supplemented with  $25 \text{ mg}\cdot\text{L}^{-1}$  kanamycin to select for the plasmid. Subsequently, bacterial cells were collected by centrifugation and resuspended in  $10 \text{ mM MgSO}_4$  to a final density of  $10^7 \text{ cfu}\cdot\text{mL}^{-1}$ . Wild-type Col-0 plants were inoculated by pressure infiltrating a

suspension of *P. syringae* pv. *tomato* DC3000(*avrRpt2*) at  $10^7$  cfu·mL<sup>-1</sup> into all fully expanded leaves of 5-week-old plants.

Bioassays with the fungal leaf pathogen *Alternaria brassicicola* strain MUCL 20297 were carried out as described by Ton *et al.* (2002). Briefly, *A. brassicicola* was grown on potato dextrose agar plates for 2 weeks at 22°C. Subsequently, conidia were collected as described by Broekaert *et al.* (1990). Five-week-old susceptible *pad3-1* plants were challenge inoculated by applying 3- $\mu$ L drops of 10 mM MgSO<sub>4</sub> containing 10<sup>6</sup> spores per mL onto all fully expanded leaves of 5-week-old plants.

### **Insect bioassays**

Tissue-chewing larvae of the small cabbage white butterfly *Pieris rapae* were reared on Brussels sprout plants (*Brassica oleracea gemmifera* cv. *Cyrus*) in a growth chamber with a 16-hr day and 8-hr night cycle (21°C; 50–70% relative humidity), as described previously (Van Poecke *et al.*, 2001). Infestation of Arabidopsis Col-0 plants was carried out by transferring five first-instar larvae of *P. rapae* to each plant using a fine paintbrush.

The population of the Western flower thrips *Frankliniella occidentalis* originated from a greenhouse infestation on chrysanthemum. This virus-free population was reared on *Phaseolus vulgaris* cv. *Prelude* pods, supplied with *Pinus* pollen, in glass jars that were placed at 25°C in a growth chamber with a 16-hr day and 8-hr night cycle as described (Kindt *et al.*, 2003). Thrips infestations were performed by transferring 20 larvae of *F. occidentalis* to each Arabidopsis Col-0 plant.

Phloem-feeding green peach aphids (*Myzus persicae*) were maintained on *Brassica chinensis* cv. *Granaat* under greenhouse conditions (25°C; 50–70% relative humidity). The 16-hr light period prevented sexual reproduction, keeping the population clonal. Arabidopsis Col-0 plants were infested with *M. persicae* by transferring 40 nymphs and apterous adults to each plant (Van Poecke *et al.*, 2003).

All insect populations used consisted of fairly immobile stages such that individuals remained on the plants to which they were transferred.

### **MeJA treatment**

Induction treatment with methyl jasmonate (MeJA) was performed by dipping 5-week-old Col-0 plants in an aqueous solution containing 0.05 mM MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands) and 0.01% of the surfactant Silwet L-77 (Van Meeuwen Chemicals B.V., Weesp, the Netherlands) as described previously (Pieterse *et al.*, 1998). Plants were harvested at 0, 1, 3, and 6 hr after induction treatment and immediately frozen in liquid nitrogen.

### ET quantification

Immediately after pathogen inoculation or transfer of insect populations to the shoots, rosettes were detached from the roots, weighed and placed individually in 35-mL gas-tight serum flasks ( $n=10$ ) that were subsequently incubated under climate chamber conditions. At different time intervals, 1-mL gas samples were withdrawn through the rubber seal. The concentration of ET was measured by gas chromatography as described by De Laat and Van Loon (1982).

### JA and SA quantification

All leaves from 20 plants per treatment were frozen in liquid nitrogen and pulverized with mortar and pestle. For each JA extraction, a sample of 1 g was taken from the frozen leaf material and transferred to a 50-mL centrifuge tube. To the frozen samples were added 100 ng of the internal standard 9,10-dihydrojasmonic acid, 10 mL of saturated NaCl solution, 0.5 mL of 1 M citric acid, and 25 mL of diethylether containing 0.005% (w/v) butylated hydroxytoluene as antioxidant. Subsequently, extraction and GC-MS quantification of JA was carried out as described by Mueller and Brodschelm (1994).

For each SA extraction, a sample of 0.5 g of ground leaf tissue was transferred to a 1.5-mL microfuge tube and 100  $\mu$ L of the internal standard ortho-anisic acid (1  $\mu$ g $\cdot$ mL<sup>-1</sup>) and 0.5 mL of 70% ethanol were added. Subsequently, extraction and quantification of SA were carried out as described by Meuwly and Métraux (1993).

### Northern blot analysis

Total RNA was extracted as described previously (Van Wees *et al.*, 1999). For northern blot analysis, 15  $\mu$ g RNA was denatured using glyoxal and DMSO (Sambrook *et al.*, 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-N+ membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. Northern blots were hybridized with gene-specific probes for *PR-1*, *PDF1.2*, *VSP2*, and *HEL* as described previously (Pieterse *et al.*, 1998). To check for equal loading, the blots were stripped and hybridized with a probe for 18S rRNA. The AGI numbers for the genes studied are At2g14610 (*PR-1*), At5g24770 (*VSP2*), At5g44420 (*PDF1.2*), and At3g04720 (*HEL*). Probe for 18S was derived from an Arabidopsis cDNA clone (Pruitt and Meyerowitz, 1986).

### Quantitative real-time PCR

Q-RT-PCR analysis was basically performed as described previously (Czechowski *et al.*, 2004). Two  $\mu$ g of RNA was digested with Turbo DNA-

free™ (Ambion, Huntingdon, United Kingdom) according to the manufacturer's instructions. To check for genomic DNA contamination, a PCR with primers designed on intron sequences of *ACT7* (At5g09810; *ACT7*-FOR; 5'-GAC ATG GAA AAG ATA TGG CAT CAC AC-3'; *ACT7*-REV; 5'-AGATCCCTTC CTG ATATCG ACATCA C-3') was carried out. Subsequently, DNA-free total RNA was converted into cDNA using oligo-dT<sub>20</sub> primers (Invitrogen, Breda, the Netherlands), 10 mM dNTPs, and SuperScript™ III Reverse Transcriptase (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Efficiency of cDNA synthesis was assessed by Q-RT-PCR using primers of the constitutively expressed gene *UBI10* (At4g05320; *UBI10*-FOR; 5' AAA GAG ATA ACA GGA ACG GAA ACA TAG T-3'; *UBI10*-REV; 5'-GGC CTT GTA TAA TCC CTG ATG AAT AAG-3'). Gene-specific primers were designed for five Arabidopsis genes, each of which showed an attacker-specific expression pattern in one of the five Arabidopsis-attacker interactions studied. The corresponding AGI numbers and primers are At1g30700, FOR 5'-TCC GTA ACC TCC GCT TCA AC-3', REV 5'-CGT GGC CTC CAC TTC TGA TT-3' (Arabidopsis-*P. syringae*); At4g26150; FOR 5' GGA TTT GGA GAC CCAGAG CA-3', REV 5'-TGG CAG CCT CCT TCT CAT CT-3' (Arabidopsis-*A. brassicicola*); At4g15210, FOR 5'-GAC GGC CTA CAA AAC GCT GT-3', REV 5'-CCA TTG TGG GAT CGG GAT AG-3' (Arabidopsis-*P. rapae*); At1g72260, FOR 5'-CTG CCC TTC CAA CCA AGC TA-3', REV 5'-TGG CAT CCA CTC ACT TGC AT-3' (Arabidopsis-*F. occidentalis*); and At5g62360, FOR 5'-CAA ACA AGC CCC AAG CTC AT-3', REV 5'-CGC ACC ATC ATT GCT GAA GT-3' (Arabidopsis-*M. persicae*). Q-RT-PCR analysis was done in optical 96-well plates with an MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands), using SYBR® Green to monitor dsDNA synthesis. Each reaction contained 1 µL of cDNA, 0.5 µL of each of the two gene-specific primers (10 pmol·µL<sup>-1</sup>), and 10 µL of 2x IQ SYBR® Green Supermix reagent (Bio-Rad, Veenendaal, the Netherlands) in a final volume of 20 µL. The following PCR program was used for all PCR reactions: 95 °C for 3 min; 40 cycles of 95 °C for 30 sec, 59.5 °C for 30 sec, and 72 °C for 30 sec. C<sub>T</sub> (threshold cycle) values were calculated using Optical System Software, version 1.0 for MyiQ™ (Bio-Rad, Veenendaal, the Netherlands). Subsequently, C<sub>T</sub> values were normalized for differences in dsDNA synthesis using the *UBI10* C<sub>T</sub> values. Normalized transcript levels of the five genes in each of the five Arabidopsis-attacker combinations were compared to those of the respective mock-treated controls and the fold change in expression level was calculated.

### GUS assays

Transgenic Arabidopsis *PDF1.2:GUS* and *PR-1:GUS* lines, containing a translational fusion of the *PDF1.2* or the *PR-1* promoter with the *uidA*

reporter gene in the Col-0 background (kindly provided by Yulia Plotnikova, Massachusetts General Hospital, Boston, USA), were grown in soil as described above. Insects were transferred to 5-week-old plants as described above. After 24 hr of caterpillar feeding or 72 hr of thrips or aphid feeding, leaf tissues were harvested and GUS activity was assessed by transferring the seedlings to GUS staining solution (1 mM X-Gluc, 100 mM NaPi buffer, pH 7.0, 10 mM EDTA, and 0.1% (v/v) Triton X-100) as described previously (Spoel *et al.*, 2003). After overnight incubation at 37°C, the leaf tissues were destained by repeated washes in 70% ethanol and evaluated for staining intensity.

### **Sample preparation and microarray data collection**

For isolation of RNA, whole rosettes were harvested at different time intervals during each Arabidopsis-attacker interaction or at several time points after MeJA treatment, and immediately frozen in liquid nitrogen. For all time points, every Arabidopsis-attacker combination, and the MeJA treatment, appropriate mock-treated plants were harvested. RNA was prepared from four biological replicates, each consisting of 5 plants, as described above and cleaned using RNeasy Plant Mini Kit columns (Qiagen Benelux BV, Venlo, the Netherlands). These replicates were pooled to reduce noise arising from biological variation. In retrospect it is now recognized that pooling RNA samples of biological replicates is not optimal. If the experiments would have been done today each biological replicate would have been used for hybridization of a GeneChip. Synthesis of cRNA probes, hybridization to GeneChips, and collection of data from the hybridized GeneChips were performed as described previously (Verhagen *et al.*, 2004; Zhu *et al.*, 2001). Hybridizations with labeled cRNAs were conducted with Arabidopsis ATH1 full-genome GeneChips (Affymetrix, Santa Clara, USA), containing a total number of 22,810 probe sets representing approximately 23,750 Arabidopsis genes (Redman *et al.*, 2004). On this GeneChip, each gene is represented by at least one 'probe set' consisting of 11 25-mer oligonucleotides. Probe preparations and GeneChip hybridizations were carried out by ServiceXS (Leiden, the Netherlands) and the Affymetrix service station of Leiden University Medical Centre (LUMC) where they passed all internal quality checks.

### **Expression profiling**

GeneChip Operating Software (GCOS) (Affymetrix, Santa Clara, USA) was used to globally normalize the expression data on each GeneChip to an average value of 200 so that hybridization intensity of all chips was equivalent. In addition, expressed genes were identified by GCOS, which uses statistical criteria to generate a 'present' or 'absent' call for genes represented by each probe set on the array. Microarray data files were then analyzed using GeneSpring

6.1 (Silicon Genetics, Redwood, CA, USA). The default settings 'Per Chip: Normalize to 50<sup>th</sup> percentile' and 'Per Gene: Normalize to specific samples' were used during the data analyses. The P-values from the Pearson correlation tests run for GeneChips that were hybridized with probes from four biological replicates of non-treated control plants ranged between 0.92 and 0.97. This is in good agreement with the high correlation coefficients previously reported for independent biological samples (Redman *et al.*, 2004), indicating that the GeneChip hybridizations and microarray data collections were performed in a technically sound manner.

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## Supplementary materials

**Supplement 1:** MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected probe sets that showed a consistent >2-fold change (up or down) 12 and 24 hr after inoculation of *Arabidopsis* Col-0 plants with the bacterial leaf pathogen *P. syringae* pv. *tomato* DC3000(*avrRpt2*).

**Supplement 2:** MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected probe sets that showed a consistent >2-fold change (up or down) 24 and 48 hr after inoculation of *Arabidopsis pad3-1* plants with the fungal leaf pathogen *A. brassicicola*.

**Supplement 3:** MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected probe sets that showed a consistent >2-fold change (up or down) 12 and 24 hr after infestation of *Arabidopsis* Col-0 plants with tissue-chewing caterpillars of the cabbage white butterfly *P. rapae*.

**Supplement 4:** MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected probe sets that showed a consistent >2-fold change (up or down) 12 and 24 hr after infestation of *Arabidopsis* Col-0 plants with larvae of the cell-content feeding Western flower thrips *F. occidentalis*.

**Supplement 5:** MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected probe sets that showed a consistent >2-fold change (up or down) 48 and 72 hr after infestation of *Arabidopsis* Col-0 plants with phloem-feeding *M. persicae* aphids.

**Supplement 6:** MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected probe sets that showed on at least two time points a consistent >2-fold change (up or down) at 1, 3, or 6 hr after treatment of *Arabidopsis* Col-0 plants with 0.05 mM MeJA.

These supplementary materials can be found at [http://www.bio.uu.nl/~fytopath/GeneChip\\_data.htm](http://www.bio.uu.nl/~fytopath/GeneChip_data.htm).

# Differential effectiveness of herbivore-induced resistance against microbial pathogens in *Arabidopsis*

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## Abstract

Plants are capable of integrating signals induced by microbial pathogens or herbivorous insects into specific inducible defense responses. Feeding by caterpillars of the cabbage white butterfly *Pieris rapae* on *Arabidopsis* is associated with an increase in the production of jasmonic acid (JA) and ethylene (ET). Prior feeding by *P. rapae* larvae triggered a systemic defense response against subsequent caterpillar attack, resulting in decreased performance of *P. rapae* larvae on systemic tissues. Wounding alone was not effective in this respect, but application of caterpillar regurgitant onto the wounds induced a similar level of systemic protection. To investigate the spectrum of effectiveness of the *P. rapae*-induced defense response, we examined the level of herbivore-induced resistance against the fungal pathogen *Alternaria brassicicola*, the bacterial pathogens *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *armoraciae*, and the viral pathogen turnip crinkle virus (TCV). Although *A. brassicicola* is sensitive to JA-dependent defense responses, *P. rapae* feeding did not result in resistance against this pathogen. *PDF1.2*, a JA-responsive marker gene for resistance against *A. brassicicola*, was suppressed by elicitors in the regurgitant of *P. rapae*, suggesting that this herbivore actively suppressed the JA-dependent defense response that is associated with resistance against this necrotrophic pathogen. In contrast, caterpillar feeding significantly reduced disease caused by *P. syringae* pv. *tomato* and *X. campestris* pv. *armoraciae*. However, this effect was apparent only locally in the caterpillar-damaged tissue and could not be mimicked by the wounding and regurgitant treatment. *Arabidopsis* mutants *jar1*, *coi1*, *ein2*, *sid2*, *eds5*, and *npr1* showed wild-type levels of *P. rapae*-induced protection against *P. syringae* pv. *tomato*, suggesting that this local, herbivore-induced defense response functions independently of JA, ET, and salicylic acid (SA). Although resistance against TCV is predominantly dependent on SA, *P. rapae*-induced defense was associated with a significant reduction of lesion development and TCV multiplication. Moreover, herbivore-induced resistance against TCV was effective both locally and systemically and could be mimicked by applying caterpillar regurgitant onto artificially wounded tissue. Analysis of SA-induced *PR-1* gene expression revealed that prior feeding by *P. rapae* primes *Arabidopsis* leaf tissue for augmented expression of SA-dependent defense responses, which may explain the observed enhanced defensive capacity against TCV. Pharmacological experiments revealed that ET acts synergistically on SA-induced *PR-1* expression, suggesting that increased production of ET in response to *P. rapae* feeding is involved in this phenomenon.

## Introduction

Plants possess a broad range of defense mechanisms to effectively combat attack by microbial pathogens and herbivorous insects. These mechanisms include pre-existing physical and chemical barriers, as well as inducible defense responses that become activated upon attack (Dicke and Hilker, 2003; Van Loon, 2000). An important question in plant defense signaling research is: how are plants capable of integrating signals induced by pathogenic micro-organisms and herbivorous insects into defenses that are specifically active against the invader encountered? The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the main players in the regulation of signaling networks involved in induced defense (Feys and Parker, 2000; Glazebrook, 2001; Kessler and Baldwin, 2002; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998; Thomma *et al.*, 2001; Van Poecke and Dicke, 2002). SA-, JA-, and ET-dependent pathways regulate defense responses that are differentially effective against specific types of attackers. Although there are exceptions (Thaler *et al.*, 2004), in general it can be stated that pathogens with a biotrophic life style are more sensitive to SA-dependent responses, whereas necrotrophic pathogens and herbivorous insects are resisted by JA/ET-dependent defenses (Glazebrook, 2005; Thomma *et al.*, 2001). For instance, in Arabidopsis induction of SA-dependent systemic acquired resistance (SAR) by avirulent *Pseudomonas syringae* pv. *tomato*, provides a significant level of protection against the biotrophic pathogen turnip crinkle virus (TCV), whereas activation of JA/ET-dependent induced systemic resistance (ISR) by non-pathogenic *Pseudomonas fluorescens* rhizobacteria is ineffective against this virus. Conversely, rhizobacteria-mediated ISR provides enhances resistance against the necrotrophic fungus *Alternaria brassicicola*, whereas pathogen-induced SAR is ineffective against this pathogen (Ton *et al.*, 2002).

The production of SA, JA, and ET varies greatly depending on the nature of the pathogen or attacking insect. The quantity, composition and timing of the hormonal blend results in the activation of a specific set of genes that eventually determines the nature of the defense response that is triggered by the attacker encountered (De Vos *et al.*, 2005). There is ample evidence that SA-, JA-, and ET-dependent defense pathways interact, either positively or negatively (Bostock, 2005; Felton and Korth, 2000; Feys and Parker, 2000; Kunkel and Brooks, 2002; Pieterse *et al.*, 2001). Global expression profiling of pathogen-infected wild-type Arabidopsis plants and a large number of SA-, JA-, or ET-signaling mutants revealed substantial cross-talk between the SA-, JA-, and ET-dependent signaling pathways (Glazebrook *et al.*, 2003). In some cases, the signaling compounds act additively on the level of resistance (Van Wees *et*

*al.*, 2000). In other cases, simultaneous activation of multiple defense signaling pathways results in antagonistic effects on pathogen and insect resistance (Bostock, 2005; Thaler *et al.*, 2002). Several key elements involved in pathway cross-talk have been identified. For instance, the SAR regulatory protein NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) has been shown to play an important role in the antagonistic effect of SA on JA-responsive gene expression (Spoel *et al.*, 2003). Furthermore, the Arabidopsis transcription factor WRKY70 was shown to act as both an activator of SA-responsive genes and a repressor of JA-inducible genes, thereby integrating signals from these two pathways (Li *et al.*, 2004). In addition, the transcription factor ETHYLENE RESPONSE FACTOR1 (ERF1) was found to integrate signals from the JA and ET pathways in activating defense-related genes that are responsive to both JA and ET (Lorenzo *et al.*, 2003). Cross-communication between defense pathways can provide a regulatory potential for activating multiple resistance mechanisms in varying combinations and may help the plant to prioritize the activation of a particular defense pathway over another, thereby providing an adapted defense against the invader encountered.

Many studies have indicated that JA and its derivatives are the most important regulators of induced resistance against herbivore attack. A classic example is the observation that following attack by larvae of *Manduca sexta*, tomato leaves accumulate JA, resulting in the activation of genes encoding proteinase inhibitor proteins that inhibit digestive serine proteinases of herbivorous insects and reduce further insect feeding (Farmer and Ryan, 1992; Howe, 2005). In agreement with this, JA-deficient tomato mutants that are affected in the *DEFENSELESS1* (*DEF1*) gene are more susceptible to attack by herbivores such as *Manduca sexta*, *Spodoptera exigua*, *Frankliniella occidentalis*, and *Tetranychus urticae* (Howe *et al.*, 1996; Li *et al.*, 2002; Thaler *et al.*, 2002). Also in Arabidopsis, genetic evidence demonstrates that jasmonates play an important role in induced defense against different types of herbivores (Ellis *et al.*, 2002; McConn *et al.*, 1997; Reymond *et al.*, 2004; Stintzi *et al.*, 2001; Stotz *et al.*, 2002; Van Poecke and Dicke, 2002; 2004). Besides being more vulnerable to herbivore attack, various Arabidopsis mutants affected in JA biosynthesis or signaling are altered in their resistance against pathogens, such as the fungi *A. brassicicola*, *Botrytis cinerea*, *Erysiphe cichoracearum*, *Erysiphe orontii*, *Fusarium oxysporum*, and *Oidium lycopersicum*, and the oomycetous pathogens *Pythium irregulare* and *Pythium mastophorum*, the bacterial pathogens *Erwinia carotovora*, *P. syringae* and *Xanthomonas campestris*, and the viral pathogen cucumber mosaic virus (Pozo *et al.*, 2005 and references herein).

The dual role of JA in herbivore and pathogen resistance prompted us to investigate the effectiveness of herbivore-induced resistance against infection by microbial pathogens. Aiming to understand how plants integrate pathogen- and

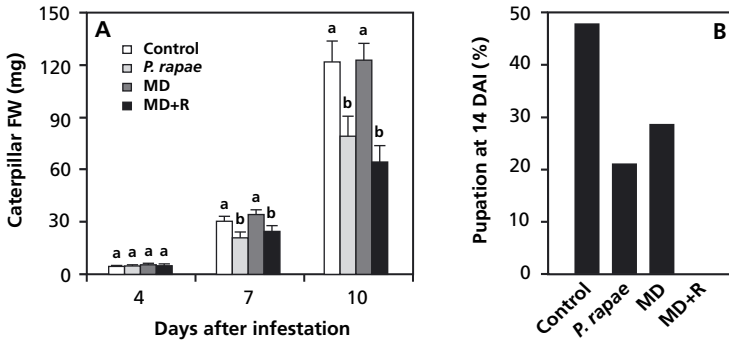
insect-induced signals into specific defense responses, we recently monitored the dynamics of SA, JA, and ET signaling in Arabidopsis after attack by a set of microbial pathogens and herbivorous insects with different modes of attack (De Vos *et al.*, 2005). Of these, the tissue-chewing caterpillar of the cabbage white butterfly (*Pieris rapae*) is a specialist on cruciferous plant species (Van Loon *et al.*, 2000). While feeding on Arabidopsis, *P. rapae* larvae induced significant levels of JA and ET and a large number of predominantly JA-responsive genes. In other studies, *P. rapae* feeding has been demonstrated to induce the expression of JA-responsive genes as well (Reymond *et al.*, 2000; 2004). Because of the nature of the response of Arabidopsis to feeding by *P. rapae* we hypothesized that caterpillar-induced resistance would be effective against pathogens that are sensitive to JA/ET-dependent defense responses, but not against pathogens that are sensitive exclusively to SA-dependent defenses.

In Arabidopsis, the dependence of induced resistance against specific pathogens on SA and/or JA and ET reflects the involvement of these signaling compounds in basal resistance that is expressed upon primary infection (Ton *et al.*, 2002). Basal resistance against the fungus *A. brassicicola* is reduced only in JA-insensitive mutants, and not in genotypes that are non-responsive to SA (Thomma *et al.*, 1998). Conversely, basal resistance against TCV is controlled exclusively by a SA-dependent pathway. Only SA-nonaccumulating NahG plants exhibited enhanced disease susceptibility to this pathogen (Kachroo *et al.*, 2000), whereas mutants affected in JA or ET signaling did not. Basal resistance against the bacterial pathogens *P. syringae* pv. *tomato* and *X. campestris* pv. *armoraciae* was found to be affected in both SA-, and in JA- and ET-response mutants (Ellis *et al.*, 2002; Pieterse *et al.*, 1998; Ton *et al.*, 2002), indicating that basal resistance against these pathogens depends on a combined action of these signals. Here, we studied whether *P. rapae*-induced resistance is differentially effective against the microbial pathogens *A. brassicicola*, *P. syringae* pv. *tomato*, *X. campestris* pv. *armoraciae*, and TCV.

## Results

### ***Pieris rapae*-induced defense against herbivore feeding**

Feeding by *P. rapae* larvae on Arabidopsis stimulates the production of JA and ET, and induces changes in the expression of a large number of defense-related genes (De Vos *et al.*, 2005; Reymond *et al.*, 2004). To verify that this induced defense response is associated with enhanced resistance against feeding by this herbivore, we monitored the fresh weight of *P. rapae* larvae on untreated and pre-infested Arabidopsis Col-0 plants. For induction of resistance, five first-instar larvae of *P. rapae* were allowed to feed for 24 hr on 5-week-



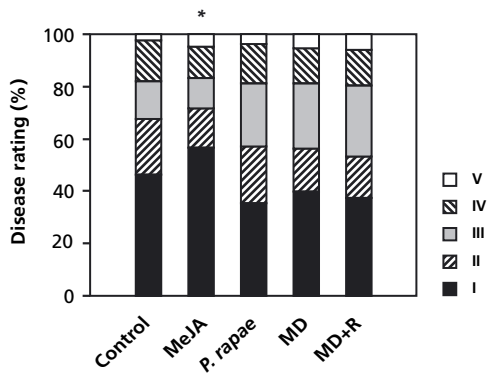
**Figure 1.** Effect of herbivore-induced resistance on *P. rapae* performance

(A) Growth of *P. rapae* larvae on herbivore- (*P. rapae*), mechanical damage- (MD), or mechanical damage- and regurgitant-induced (MD+R) Arabidopsis Col-0 plants. Freshly hatched *P. rapae* larvae were transferred onto non-induced (Control) and induced plants 24 hr after the start of the induction treatment. Larval fresh weight (FW) was measured after 4, 7, and 10 days of feeding. The values presented are means ( $\pm$ SE) of 20 larvae that received the same treatment. Different letters indicate statistically significant differences between treatments (Fisher's LSD test;  $\alpha=0.05$ ).

(B) Percentage of *P. rapae* larvae ( $n=20$ ) that developed into pupae within 14 days after infestation (DAI).

old Col-0 plants. Subsequently, the caterpillars were removed and replaced by a fresh first-instar larva of which the fresh weight was monitored over a 10-day period. Figure 1A shows that the increase in weight of the *P. rapae* larvae was significantly reduced on pre-infested plants. To investigate whether herbivore-induced resistance could be mimicked by wounding, Arabidopsis leaves were mechanically damaged with a needle and tested for enhanced resistance against *P. rapae* feeding. Moreover, mechanically damaged leaves were supplemented with regurgitant that was collected from other *P. rapae* larvae that had fed on Col-0 plants. Whereas wounding alone did not reduce larval weight gain, application of regurgitant onto the wounds induced similar levels of herbivore resistance as *P. rapae* feeding did (Fig. 1A).

To investigate whether the reduced larval performance on induced plants affected the development of the larvae into pupae, the percentage of the larvae that reached pupation was assessed 14 days after transfer of the first-instar larvae onto the Arabidopsis plants. Figure 1B shows that the number of caterpillars that developed into pupae was clearly lower in herbivore- and wound/regurgitant-treated plants. These results indicate that *P. rapae* feeding induces a defense response that inhibits growth and development of other larvae that subsequently feed on the leaves. This herbivore-induced resistance can be mimicked by applying regurgitant of *P. rapae* onto the wound sites of mechanically damaged Arabidopsis leaves.

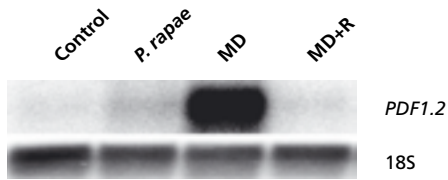


**Figure 2.** Effectiveness of herbivore-induced resistance against *A. brassicicola*

To trigger herbivore-induced resistance, 3 first-instar larvae of *P. rapae* were allowed to feed for 24 hr on mutant *pad3-1* plants, which is a susceptible host for this pathogen. MeJA-induced resistance was elicited by dipping the leaves in a solution containing 0.1 mM MeJA, 24 hr before challenge. Plants were challenge inoculated with *A. brassicicola* when 5 weeks old, and scored for final disease symptoms 6 days later. Disease severity is expressed on the basis of symptoms severity and lesion size (increasing severity from I to V; see Materials and Methods for details). Asterisks indicate statistically significant different distributions within the disease-severity classes compared with the non-induced control treatment (Chi-square,  $\alpha=0.05$ ;  $n=15$ ).

### Herbivore-induced resistance is not effective against *Alternaria brassicicola*

Because feeding by *P. rapae* increased the production of both JA and ET (De Vos *et al.*, 2005), we hypothesized that the resulting resistance would also be effective against the necrotrophic fungal pathogen *A. brassicicola*. Wild-type Arabidopsis Col-0 plants are highly resistant to *A. brassicicola* infection. However, the phytoalexin-deficient mutant *pad3-1* is substantially more susceptible (Thomma *et al.*, 1999), and has been used successfully to study induced resistance against this pathogen (Ton *et al.*, 2002). To trigger herbivore-induced resistance, three *P. rapae* larvae were allowed to feed on *pad3-1* plants for 24 hr. As a positive control, *pad3-1* plants were treated with 0.1 mM MeJA, which has been shown to induce resistance against *A. brassicicola* (Ton *et al.*, 2002). Subsequently, non-induced, MeJA-treated, and herbivore-damaged plants were inoculated with *A. brassicicola*. In non-induced plants, necrotic lesions started to appear within 2 to 3 days after inoculation and progressed into typical spreading lesions that were surrounded by extensive chlorosis. By 6 days after inoculation, the leaves were extensively damaged and sporulation of the pathogen was evident. Exogenous application of MeJA 24 hr prior to challenge inoculation resulted in a significant reduction in disease severity. However, although JA levels were increased up to 10-fold in *P. rapae*-induced plants (De Vos *et al.*, 2005), no enhanced resistance against *A. brassicicola* infection could be observed in these plants (Fig. 2). Moreover,



**Figure 3. Herbivore-induced suppression of *PDF1.2* gene expression**

Northern blot analysis of the JA/ET-responsive marker gene *PDF1.2* 24 hr after infestation with first-instar larvae of *P. rapae*, mechanical damage (MD), or mechanical damage followed by treatment with caterpillar regurgitant (MD+R). Equal loading of RNA samples was checked using a probe for 18S rRNA.

neither wounding nor application of *P. rapae* regurgitant onto the wounds resulted in enhanced resistance. It must, therefore, be concluded that *P. rapae*-induced resistance is not effective against *A. brassicicola*.

### **Suppression of JA-dependent defense responses by *P. rapae* feeding**

The primary defense response of *Arabidopsis* against *P. rapae* and *A. brassicicola* involves an increase in the production of JA and the activation of a large set of JA-responsive genes (De Vos *et al.*, 2005). However, the overlap between the JA-responsive changes that were induced by *P. rapae* and *A. brassicicola* is relatively small (up to 9%) (De Vos *et al.*, 2005). This suggests that although JA is a main signal, the JA-mediated defense responses against these attackers are regulated differentially. *P. rapae* is a specialist herbivore on cruciferous plants and may have developed the capacity to suppress plant defense responses. To investigate whether it can suppress JA-dependent defense responses that are associated with resistance against *A. brassicicola*, we monitored the expression of the JA-responsive marker gene *PDF1.2*. *PDF1.2* codes for PLANT DEFENSIN1.2 that inhibits growth of *A. brassicicola* *in vitro* (Penninckx *et al.*, 1996), and is associated with enhanced resistance against this pathogen (Penninckx *et al.*, 2003). Figure 3 shows that *PDF1.2* is not activated after feeding by *P. rapae* for 24 hr, even though JA levels are significantly increased (data not shown). In contrast, *PDF1.2* mRNA levels were strongly increased 24 hr after mechanical damage of the leaves. Application of *P. rapae* regurgitant onto the wound sites prevented *PDF1.2* transcript accumulation. This active suppression of JA-responsive gene expression by *P. rapae* can explain why *P. rapae*-induced resistance is not effective against *A. brassicicola*.

### ***P. rapae*-induced resistance is locally effective against two bacterial leaf pathogens**

Previously, *Arabidopsis* mutants affected in either SA, JA, or ET signaling were demonstrated to be affected in the level of resistance to the bacterial pathogens *X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato* (Ellis *et al.*, 2002; Pieterse *et al.*, 1998; Ton *et al.*, 2002), implying a role for all three signals in the defense against these pathogens. To investigate the effectiveness

of herbivore-induced resistance against both these bacterial pathogens, Col-0 plants were exposed to *P. rapae* feeding for 24 hr, and subsequently challenge inoculated with *X. campestris* pv. *armoraciae* or *P. syringae* pv. *tomato*. Disease symptoms on *P. rapae*-damaged leaves were less severe than on non-damaged leaves of the same plants. Therefore, damaged (local) and non-damaged (systemic) leaves were assessed separately. Figure 4A and 4B show that *P. rapae* feeding induced a significant level of resistance against both *X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato* in the *P. rapae*-damaged, local leaves, but not in the undamaged, systemic leaves.

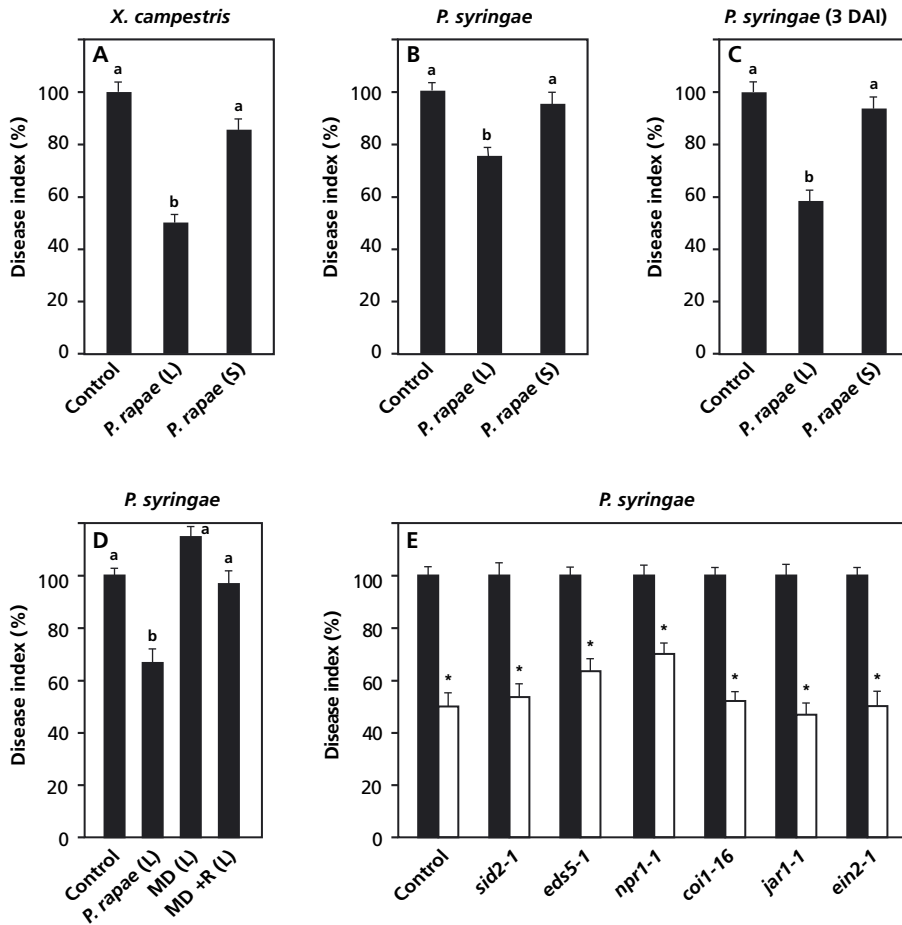
Because *P. rapae*-induced plants were challenge inoculated with the bacterial pathogens immediately after removal of the caterpillars, the time between induction and expression of resistance may have been too short to mount an effective systemic effect. To clarify this point, *P. rapae*-induced plants were challenge inoculated with *P. syringae* pv. *tomato* 3 days after removal of the caterpillars. *P. rapae*-damaged leaves mounted a significant level of local resistance against *P. syringae* pv. *tomato* infection (Figure 4C) that was also expressed as a reduction in bacterial growth in the leaves (data not shown). However, again resistance was not expressed systemically, even though the leaves had been allowed more time to mount a defense response. It can, thus, be concluded that *P. rapae* feeding enhances the level of resistance against both bacterial pathogens, but that this resistance is localized to the herbivore-damaged tissues and is not expressed systemically.

To investigate whether elicitors of herbivore-induced local resistance against *P. syringae* pv. *tomato* are present in the regurgitant of *P. rapae*, we applied regurgitant onto the wounded sites of mechanically damaged leaves and assessed the level of induced protection against *P. syringae* pv. *tomato*. Figure 4D shows that neither mechanical damage, nor a combination treatment of mechanical damage and *P. rapae* regurgitant mimicked the resistance reaction that was induced upon caterpillar feeding.

To study the role of SA, JA, and ET in *P. rapae*-induced local resistance against *P. syringae* pv. *tomato*, we tested different Arabidopsis genotypes that are affected in either SA (*sid2-1*, *eds5-1*, *npr1-1*), JA (*coi1-16*, *jar1-1*), or ET (*ein2-1*) signaling. Figure 4E shows that all genotypes tested were fully capable of expressing caterpillar-induced resistance against *P. syringae* pv. *tomato*, suggesting that the observed local resistance functions independently of SA, JA, and ET signaling.

### **Local and systemic effects of herbivore-induced resistance against TCV**

TCV is virulent on most Arabidopsis accessions, including Col-0 (Simon *et al.*, 1992), but avirulent on accession Dijon (Di-0), which develops a hypersensitive response (HR) and does not allow systemic spreading of



**Figure 4.** Herbivore-induced resistance against *X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato*

Herbivore-induced resistance was triggered in Col-0 plants by allowing *P. rapae* to feed on the leaves for 24 hr. Immediately after removal of the caterpillars or 3 days later (= 3 days after infestation (DAI)), plants were challenge inoculated with either *X. campestris* pv. *armoraciae* or *P. syringae* pv. *tomato* by dipping the leaves in a bacterial suspension containing  $10^8$  or  $5.10^6$  CFU.mL, respectively. Three days after challenge inoculation, the percentage of diseased leaves per plant was determined and the disease index was calculated relative to challenged control plants (set at 100%). To discriminate between local (L) and systemic (S) effects, *P. rapae*-damaged and undamaged leaves on the same plants were scored separately. The values presented are means ( $\pm$ SE) of 20-25 plants that received the same treatment. Different letters indicate statistically significant differences between treatments (Fisher's LSD test;  $\alpha=0.05$ ).

**A.** *P. rapae*-induced resistance against *X. campestris* pv. *armoraciae*.

**B.** *P. rapae*-induced resistance against *P. syringae* pv. *tomato*.

**C.** Effect of a 3 day interval between induction and challenge inoculation on *P. rapae*-induced resistance against *P. syringae* pv. *tomato*.

**D.** Effect of mechanical damage (MD) and mechanical damage in combination with *P. rapae* regurgitant (MD+R) on the level of resistance against *P. syringae* pv. *tomato*.

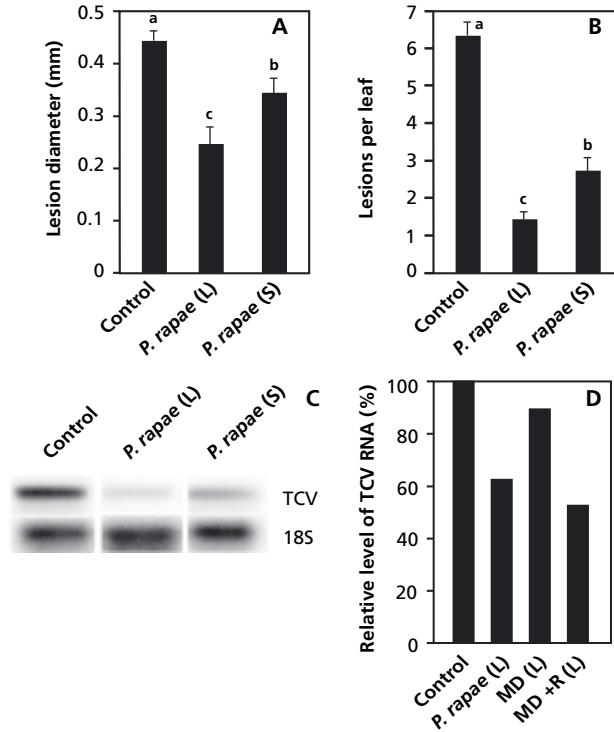
**E.** *P. rapae*-induced resistance against *P. syringae* pv. *tomato* in Arabidopsis defense signaling mutants. The absolute proportions of diseased leaves of uninduced control plants were 41% (A), 60% (B), 62% (C), 79% (D) and 72% (Col-0), 85% (*sid2-1*), 75% (*eds5-1*), 77% (*npr1-1*), 44% (*coi1-16*), 78% (*jar1-1*), and 78% (*ein2-1*) (E).

the pathogen (Dempsey *et al.*, 1997; Simon *et al.*, 1992). To investigate the effectiveness of *P. rapae*-induced resistance against TCV, Di-0 plants were exposed to *P. rapae* feeding for 24 hr and subsequently challenge inoculated with TCV. Five days later, the level of induced protection was examined by determining lesion size and TCV RNA levels in control and *P. rapae*-induced plants. Caterpillar feeding resulted in a significant reduction in lesion size (Fig. 5A), and a strong reduction in the number of lesions per leaf (Fig. 5B). Moreover, TCV RNA accumulated to much lower levels in the *P. rapae*-induced than in control plants (Fig. 5C). The effects on lesion development and TCV multiplication were apparent in herbivore-damaged and non-damaged leaves of herbivore-induced plants, indicating that *P. rapae*-induced resistance against TCV is effective both locally and systemically.

To investigate whether elicitors of *P. rapae* are involved in herbivore-induced resistance against TCV, we examined whether mechanical damage or a combination of mechanical damage and regurgitant treatment affect TCV RNA multiplication. Figure 5D shows that mechanical damage alone did not result in a reduction of TCV RNA levels. However, application of *P. rapae* regurgitant onto the wounded sites resulted in a reduction in TCV RNA levels similar to what was observed upon caterpillar feeding, indicating that factors in the regurgitant of *P. rapae* are responsible for herbivore-induced resistance against TCV.

#### ***P. rapae*-induced resistance is associated with priming for SA-dependent defense responses**

In Di-0, exogenous application of 2,6-dichloroisonicotinic acid (INA; a functional analogue of SA) reduces the lesion size caused by TCV infection and inhibits viral multiplication (Kachroo *et al.*, 2000), whereas MeJA and the ET precursor 1-aminocyclopropane-1-carboxylate (ACC) have no effect in this respect (Ton *et al.*, 2002). Hence, TCV is sensitive to SA-dependent defenses, whereas JA/ET-dependent defense responses are ineffective. Although feeding by *P. rapae* larvae does not trigger increased SA levels (De Vos *et al.*, 2005), it did induce local and systemic resistance against TCV. This prompted us to investigate whether feeding by *P. rapae* primes the plant tissue for enhanced expression of SA-responsive genes following TCV infection. TCV infection induces *PATHOGENESIS RELATED-1* (*PR-1*) gene expression in a SA-dependent manner (Kachroo *et al.*, 2000). Therefore, we analyzed the expression of the SA-responsive *PR-1* gene in control and SA-treated leaves of uninfested and *P. rapae*-infested Di-0 and Col-0 plants. In uninfested Di-0 and Col-0 plants, *PR-1* transcripts accumulated within 24 hr after SA treatment (Fig. 6). However, in *P. rapae*-infested plants of both accessions, increase levels of *PR-1* mRNA were already detectable at 6 hr after SA treatment and *PR-1* transcript levels had accumulated further by 24 hr. These results indicate that herbivore



**Figure 5.** Herbivore-induced resistance against TCV

Herbivore-induced resistance was triggered in Di-0 plants by allowing *P. rapae* to feed on the leaves for 24 hr. Immediately after removal of the caterpillars plants were challenge inoculated with TCV by rubbing 3- $\mu$ l droplets of TCV RNA (0.1  $\mu$ g.  $\mu$ L<sup>-1</sup>) in bentonite buffer onto 3 *P. rapae*-damaged, local (L) leaves, and 3 undamaged, systemic (S) leaves. Five days after challenge, average lesion size, average number of lesions per leaf, and TCV RNA levels were determined. Different letters indicate statistically significant differences between treatments (Fisher's LSD test;  $\alpha=0.05$ ).

**A.** Local and systemic effects of *P. rapae*-induced resistance on TCV lesion size. The values presented are means ( $\pm$ SE) of all lesions measured on 15 plants that received the same treatment.

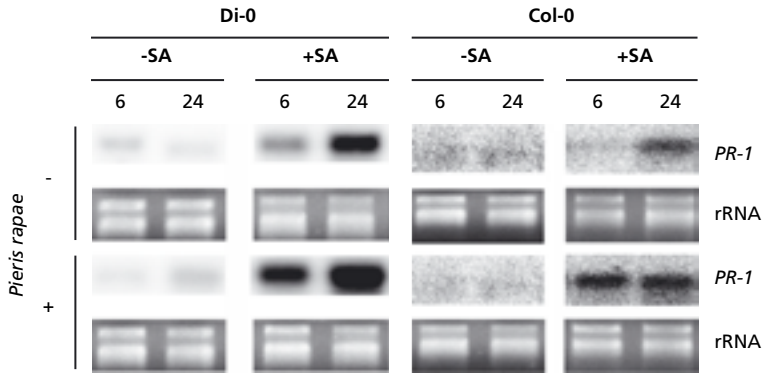
**B.** Local and systemic effects of *P. rapae*-induced resistance on the number of lesions per leaf. The values presented are means ( $\pm$ SE) from 15 plants that received the same treatment.

**C.** Accumulation of TCV RNA 5 days after challenge inoculation of control and *P. rapae*-induced Di-0 plants. Blots were hybridized with a TCV-specific probe. Equal loading of RNA samples was checked using a probe for 18S rRNA.

**D.** Effect of mechanical damage (MD) and mechanical damage in combination with *P. rapae* regurgitant (MD+R) on TCV RNA accumulation. Signal intensities of TCV RNA on the RNA blots were quantified using a Phosphor Imager, normalized for equal levels of 18S rRNA, and compared to the normalized TCV RNA levels in the uninduced control plants (set at 100%).

feeding primed the plant tissue for augmented expression of the SA-responsive *PR-1* gene.

*P. rapae* feeding is associated with the production of both ET and JA (De Vos *et al.*, 2005). Both hormones have been demonstrated to modulate SA-dependent defense responses (Pieterse *et al.*, 2001). To investigate whether ET or JA play a role in priming of *P. rapae*-induced tissue for augmented



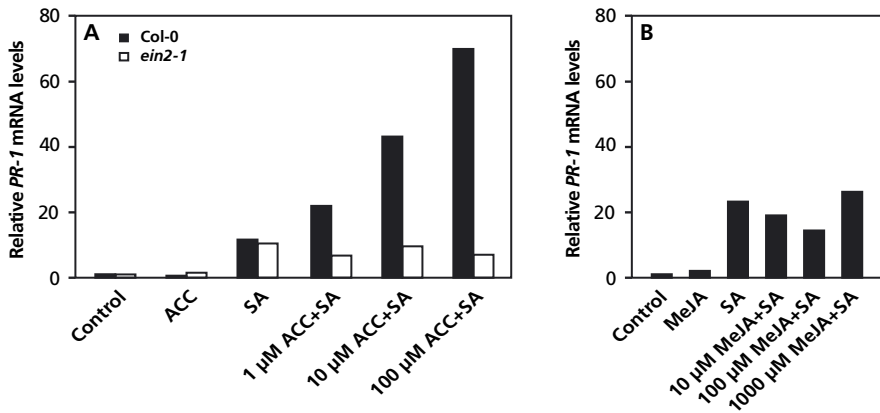
**Figure 6.** *P. rapae*-induced priming of SA-induced *PR-1* gene expression

*P. rapae* was allowed to feed on Di-0 and Col-0 plants for 24 hr. After removal of the caterpillars, uninfested and *P. rapae*-infested plants were either treated with 1 mM SA or not. Six and 24 hr later, the leaf tissue was harvested for RNA blot analysis of *PR-1* mRNA. Equal loading of RNA samples was checked by staining rRNA bands with ethidium bromide.

SA-dependent defense responses, we analyzed the effect of ACC and MeJA on SA-induced expression of *PR-1*. Figure 7A shows the changes in *PR-1* gene expression in Col-0 plants upon treatment with ACC, SA, or a combination of SA and increasing concentrations of ACC. Exogenous application of SA resulted in a 11-fold increase in *PR-1* transcript levels, whereas the ACC treatment had no effect. In the combination treatments, ACC enhanced the level of SA-induced *PR-1* expression in a dose-dependent manner. This additive effect of ACC on SA-induced *PR-1* expression was not apparent in the ET-insensitive mutant *ein2-1* (Fig. 7A), indicating that ET primed the leaf tissue for enhanced expression of *PR-1* by SA. Figure 7B shows a similar analysis of *PR-1* gene expression upon treatment with MeJA, SA, or a combination of SA and MeJA. Alone, MeJA did not induce *PR-1* gene expression. In the combination treatments, increasing concentrations of MeJA did not significantly affect SA-induced *PR-1* mRNA levels, indicating that MeJA has neither an additive, nor an antagonistic effect on this SA-induced defense response.

## Discussion

Little is known about how plants coordinate attacker-induced signals into specific defense responses. Recently, we studied the signal signature and the whole-genome expression profile of Arabidopsis upon attack by pathogens and insects with very different modes of action (De Vos *et al.*, 2005). In four of the five Arabidopsis-attacker combinations tested, JA played an important role in the differential regulation of a large proportion of the attacker-activated/



**Figure 7.** Effect of ACC and MeJA on SA-induced expression of *PR-1*

Analysis of the SA-responsive *PR-1* gene in wild-type Col-0 and mutant *ein2-1* plants. Five-week-old plants were treated with 10 μM ACC, 100 μM MeJA, 1 mM SA, or a combination of 1 mM SA and increasing concentrations of either ACC or MeJA. Twenty-four hours after chemical treatment, the leaf tissue was harvested for RNA blot analysis of *PR-1* mRNA. To check for equal loading, RNA blots were stripped and hybridized with a probe for 18S rRNA. Signal intensities of *PR-1* mRNA on the RNA blots were quantified using a Phosphor Imager, normalized for equal levels of *TUB* mRNA, and compared to the normalized *PR-1* mRNA levels in the untreated control plants (Control; set at 1).

**A.** Effect of ACC on SA-induced *PR-1* expression in Col-0 and ethylene-insensitive *ein2-1* plants.

**B.** Effect of MeJA on SA-induced *PR-1* expression in wild-type Col-0 plants.

repressed genes (i.e., in the interactions of *Arabidopsis* with *P. syringae* pv. *tomato*, *A. brassicicola*, *P. rapae*, and the Western flower thrips *Frankliniella occidentalis*). Nevertheless, the vast majority of the JA-responsive changes were specific for each plant-attacker combination. Evidently, signal molecules such as JA play an important role in the primary response of the plant to pathogen and insect attack, but the final outcome of the resistance reaction is shaped by so far unidentified additional factors.

### Herbivore-induced resistance against microbial pathogens

Feeding of *P. rapae* caterpillars on *Arabidopsis* is associated with an enhanced production of both JA and ET, whereas the levels of SA remain unaltered (De Vos *et al.*, 2005). Upon *P. rapae* feeding, *Arabidopsis* plants mount a defense response that is effective against subsequent infestation by the same herbivore (Fig. 1A and 1B), confirming previous findings in other plant species (Howe, 2005; Kessler and Baldwin, 2002). Because of the dual role of JAs in both pathogen and insect resistance, we investigated whether *P. rapae* feeding triggers cross-resistance against microbial pathogens. Our data show that herbivore-induced resistance in *Arabidopsis* is ineffective against the necrotrophic pathogen *A. brassicicola* (Fig. 2), locally effective against the bacterial pathogens *X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato*

(Fig. 4), and locally and systemically effective against TCV (Fig. 5). Mechanical damage alone was ineffective, but in combination with *P. rapae* regurgitant the effectiveness of *P. rapae*-induced resistance could be mimicked in most, but not all, cases.

### ***A. brassicicola***

Because *A. brassicicola* has been demonstrated to be sensitive to JA-dependent defense responses (Thomma *et al.*, 1998; Ton *et al.*, 2002), the lack of cross-resistance against this necrotrophic fungal pathogen was unexpected. Whole-genome expression profiling revealed that, although about 50% of all the *P. rapae*- or *A. brassicicola*-induced genes are regulated by JA, less than 10% of these JA-responsive gene sets overlap (De Vos *et al.*, 2005). Hence, whereas JA may be an important primary signal in the defense response that is activated upon attack by either *P. rapae* or *A. brassicicola*, the final outcome of the resistance reaction is highly divergent, and in the case of *P. rapae* feeding is only effective against the herbivore, and not against the necrotrophic fungus.

Interestingly, wounding alone induced the expression of the JA-responsive gene *PDF1.2*, which has been demonstrated to be a good marker gene for resistance against *A. brassicicola* (Penninckx *et al.*, 1996; 2003), but damage caused by *P. rapae* feeding did not (Fig. 3). Application of *P. rapae* regurgitant onto the wounds resulted in a suppression of wound-induced *PDF1.2* expression. These results suggest that *P. rapae*-derived elicitors are involved in the suppression of JA-dependent defense responses that are associated with resistance against *A. brassicicola*. Recently, Lorenzo *et al.* (2004) demonstrated that the transcription factors AtMYC2 and ERF1 antagonistically regulate differential sets of JA-responsive genes that are activated in response to herbivore and pathogen attack. They showed that AtMYC2 represses JA-responsive genes that are involved in defense against pathogens (e.g. *PDF1.2*), whereas ERF1 acts as a positive regulator in this respect. Expression profiling studies indeed revealed that *AtMYC2* is up-regulated upon feeding by *P. rapae*, whereas *ERF1* is not (De Vos *et al.*, 2005; Reymond *et al.*, 2004). This supports the notion that *AtMYC2* serves as an important regulator in discriminating between different JA-regulated defense responses Lorenzo *et al.* (2004).

### ***X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato***

*P. rapae*-induced resistance was effective against the bacterial pathogens *X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato*. However, enhanced resistance could only be observed in caterpillar-damaged tissue, and not systemically in undamaged leaves of *P. rapae*-infested plants. While application of *P. rapae* regurgitant onto mechanically damaged sites mimicked the herbivore-induced effect on *P. rapae* performance (Fig. 1), it had no effect on the level

of resistance against *P. syringae* pv. *tomato* (Fig. 1D). Hence, herbivore-induced defense responses seem to branch into at least two distinct types of resistance: one that affects *P. rapae* performance, and another that is effective against the bacterial pathogens. This is supported by the fact that *P. rapae* performance is affected in the JA-insensitive *coi1* mutant (Reymond *et al.*, 2004), whereas the *P. rapae*-induced resistance against *P. syringae* pv. *tomato* is still functional in this mutant (Fig. 4E). Another JA-response mutant, *jar1-1*, as well as the SA- and ET-signaling mutants *sid2-1*, *eds5-1*, *npr1-1*, and *ein2-1* mounted wild-type levels of resistance against *P. syringae* pv. *tomato* in herbivore-induced leaves, suggesting that this type of induced resistance does not require all three regulators simultaneously. SA, JA, and ET have all been implicated in the regulation of induced resistance against *P. syringae* pv. *tomato* (Ellis *et al.*, 2002; Pieterse *et al.*, 1998; Ton *et al.*, 2002). Hence, the effectiveness of *P. rapae*-induced resistance against this pathogen in the signaling mutants is not unexpected. Previously, Stout *et al.* (1999) showed that damage caused by the corn earworm *Helicoverpa zea* induced resistance in tomato against *P. syringae* pv. *tomato*, suggesting that herbivore-induced resistance against this bacterial pathogen is effective in different plant species.

### TCV

Prior infestation with *P. rapae* inhibited multiplication of TCV and significantly reduced the size and number of TCV lesions (Fig. 5). The effect of *P. rapae*-induced resistance against TCV was apparent not only locally in herbivore-damaged tissue, but also systemically in undamaged leaves of infested plants. The inhibition of TCV multiplication could be mimicked by application of *P. rapae* regurgitant, suggesting that elicitors in the regurgitant of *P. rapae* are responsible for the activation of this systemic defense response. Resistance against this biotrophic pathogen is regulated predominantly by SA (Kachroo *et al.*, 2000; Ton *et al.*, 2002). *P. rapae* feeding is not accompanied by changes in SA levels (De Vos *et al.*, 2005). In this study we demonstrated that *P. rapae* feeding primes the plant tissue for augmented, SA-inducible gene expression (Fig. 6). Moreover, we show that ET acts synergistically on the level of SA-induced *PR-1* gene expression, confirming previous findings (Lawton *et al.*, 1994), whereas MeJA does not (Fig. 7). Hence, the increased production of ET upon herbivore feeding can sensitize the tissue to respond to SA and may contribute to the enhanced resistance against TCV. In this scenario, herbivore-induced ET primes the leaf tissue for augmented SA-dependent defenses, thereby providing an enhanced defensive capacity towards pathogens, such as TCV, that trigger SA-dependent defense responses upon infection. Priming for augmented expression of pathogen-induced defense responses is implicated in different types of chemically- and microbially-induced resistance (Conrath *et*

*al.*, 2002; Newman *et al.*, 2002; Ton *et al.*, 2005; Verhagen *et al.*, 2004). Here we show that herbivore feeding induces a similar alarmed state leading to cross-resistance against a viral pathogen.

### **Mechanical damage and *P. rapae* regurgitant**

In this study we demonstrated that herbivore-induced resistance against *P. rapae* feeding and TCV could not be mimicked by mechanical damage alone. However, application of regurgitant of *P. rapae* to the wounded sites resulted in similar levels of resistance as did prior infestation with *P. rapae* (Fig. 1B and 5D). Similarly, the suppression of *PDF1.2* gene expression, observed in *P. rapae*-damaged leaves, could be mimicked by applying *P. rapae* regurgitant to artificially damaged sites (Fig. 3). Puncturing leaves or scratching the leaf surface is a common way to imitate feeding by herbivores. However, mechanically damaging leaf tissue only partially mimics the response of plants to herbivore feeding. For instance, artificially wounded leaf tissues do not produce the same blends of volatiles as do leaf tissues that have been injured by feeding herbivores (Mattiacci *et al.*, 1995; Van Poecke *et al.*, 2002). Using a mechanical caterpillar, named MecWorm, Mithöfer *et al.* (2005) demonstrated that computerized continuous damage resembles the insect's feeding process much better, leading to the production of a volatile blend that is more similar than wounding at a single time point. Evidently, the dynamics of wounding inflicted by grazing herbivores influence the nature of the induced plant defense response to a large extent.

Other factors that influence the wound response upon insect feeding are elicitors that are released by the herbivore during feeding. Application of regurgitant from feeding herbivores to mechanically damaged sites has been demonstrated to mimic specific herbivore-induced defense responses. For instance, cabbage leaves that are artificially damaged and subsequently treated with regurgitant of *Pieris brassicae* caterpillars, release a volatile blend similar to that of herbivore-damaged plants, leading to the attraction of parasitic wasps that attack the herbivores (Mattiacci *et al.*, 1995). Insect-derived compounds, such as the enzyme  $\beta$ -glucosidase and fatty acid-amino acid conjugates, such as volicitin, have been identified as potent elicitors of volatile production in different plant-herbivore interactions (Halitschke *et al.*, 2001; Mattiacci *et al.*, 1995; Turlings *et al.*, 2000). Besides insect-derived elicitors, caterpillar regurgitant also contains high levels of plant-derived molecules, including JA, the jasmonate precursor 12-oxo-phytodienoic acid (OPDA), and dinor oxo-phytodienoic acid (dnOPDA) (Reymond *et al.*, 2004), which have been shown to play a critical role in herbivore resistance in *Arabidopsis* (Stintzi *et al.*, 2001).

	<i>H. parasitica</i>	<i>E. carotovora</i> pv. <i>carotovora</i>	<i>P. syringae</i> pv. <i>tomato</i>	<i>X. campestris</i> pv. <i>armoraciae</i>	TCV	<i>A. brassicicola</i>	<i>B. cinerea</i>
ISR	+/-	+	+	+	-	+	+
SAR	+	+	+	+	+	-	-
<i>P. rapae</i> IR		+	+	+	-		

**Figure 8.** Effectiveness of ISR, SAR, and *P. rapae*-induced resistance against different types of pathogens in *Arabidopsis*.

Our study of the spectrum of effectiveness of *P. rapae*-induced resistance demonstrates that components of the caterpillar regurgitant play an important role in the activation of resistance against the insect itself and against *P. rapae* and TCV. However, the nature of the elicitor(s) involved remains to be elucidated. Neither application of *P. rapae* regurgitant onto artificially damaged leaves, nor wounding alone induced resistance against the bacterial pathogens *X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato* (Fig. 4D). Hence, elicitors in the regurgitant of *P. rapae* are not involved in the defense response against these pathogens.

Previously, we demonstrated that pathogen-induced SAR is effective against pathogens that in non-induced plants are resisted through SA-dependent defenses, whereas rhizobacteria-mediated ISR is effective against pathogens that in non-induced plants are resisted through JA/ET-dependent defenses (Ton *et al.*, 2002; Van Pelt and Pieterse, unpublished results). This suggests that SAR and ISR constitute a reinforcement of extant SA- or JA/ET-dependent basal defense responses, respectively (summarized in Fig. 8). Here we showed that herbivore feeding induces cross-resistance against several microbial pathogens. However, the observed spectrum of effectiveness was clearly different from that which was predicted on the basis of the known effectiveness of JA and ET that are produced upon feeding by *P. rapae*. We expected enhanced resistance against the necrotrophic fungus *A. brassicicola*, because this pathogen has been shown to be sensitive to JA-dependent defenses. On the other hand, we expected no effect on the level of resistance against the biotrophic pathogen TCV, because resistance against this pathogen has been demonstrated to be regulated by SA, which is not produced during feeding by *P. rapae*. Both expectations appeared to be false, because other regulating factors influenced the outcome of the defense

response. We provided evidence that elicitors in the caterpillar regurgitant actively suppress a branch of the JA response that is involved in pathogen resistance (exemplified by *PDF1.2* expression), thereby possibly prioritizing JA-dependent defenses that are directed against insect feeding. In addition, we confirmed that ET acts synergistically on SA-inducible defenses, suggesting that herbivore-induced ET production may be involved in the observed enhanced resistance against TCV. Through evolution plants developed sophisticated defensive strategies to perceive attack by microbial pathogens and herbivorous insects, and to translate that perception into an appropriate defense response. Our study demonstrates that the defense response that is triggered upon insect feeding is surprisingly complex. Synergistic and antagonistic effect of cross-talk between, and within SA-, JA-, and ET-dependent signaling pathways play an important role in determining the final outcome of the resistance reaction. Understanding the complexity of the coordinated cellular responses involved in this process is a major challenge for future research.

## Materials and Methods

### Cultivation of plants

Seeds of *Arabidopsis thaliana* accessions Col-0, Di-0 and the Col-0 mutants *pad3-1* (Glazebrook and Ausubel, 1994), *jar1-1* (Staswick *et al.*, 1992), *coi1-16* (Ellis and Turner, 2002), *ein2-1* (Guzmán and Ecker, 1990), *sid2-1* (Nawrath and Métraux, 1999), *eds5-1* (Wildermuth *et al.*, 2001), and *npr1-1* (Cao *et al.*, 1994) were sown in quartz sand. Two-week-old seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min. Plants were cultivated in a growth chamber with a 8-hr day (200  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  at 24°C) and 16-hr night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing 10  $\mu\text{M}$  Sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

### Herbivore-induction, wounding and regurgitant treatment

Tissue-chewing larvae of the small cabbage white butterfly *Pieris rapae* were reared on Brussels sprout plants (*Brassica oleracea gemmifera* cv. Cyrus) in a growth chamber with a 16-hr day and 8-hr night cycle (21°C; 50–70% relative humidity), as described previously (De Vos *et al.*, 2005; Van Poecke *et al.*, 2001). To trigger herbivore-induced resistance, 5-week-old *Arabidopsis* plants were infested by transferring 3–5 freshly hatched first-instar larvae (L1) onto each plant. The larvae were allowed to feed for 24 hr after which they

were removed. During the feeding period, most of the larvae remained on the leaf to which they had been transferred.

The effect of wounding was assessed by mechanically damaging of the leaf tissue. Three small holes (1 mm diameter) were punctured in each of 5 leaves per plant using a sterile needle. To study the effect of *P. rapae* regurgitant, 1  $\mu$ L of freshly collected regurgitant of *P. rapae* was divided over the 3 punctured holes of each mechanically damaged leaf. Regurgitant was collected from L4-L5 larvae that were allowed to feed on uninduced Col-0 plants as described (Mattiacci *et al.*, 1995). Twenty-four hr after the start of the induction treatments, non-induced and induced plants were challenged with *P. rapae*, or one of the microbial pathogens.

### ***Pieris rapae* assays**

To study the effect of herbivore feeding and wounding on *P. rapae* performance, a single freshly hatched first-instar larva was transferred to each of 20 non-induced or induced Col-0 plants. At 4, 7, and 10 days, the fresh weights of the larvae were determined. After 10 days, the first larvae started to pupate. Therefore, fresh weight was determined only up to 10 days of feeding. To examine effects on caterpillar development, the percentage of caterpillars that had pupated within 14 days after hatching was determined.

### ***Alternaria brassicicola* bioassays**

Bioassays with the fungal pathogen *A. brassicicola* MUCL 20297 were performed essentially as described by Ton *et al.* (2002). Briefly, *A. brassicicola* was grown on potato dextrose agar plates for 2 weeks at 22°C. Conidia were harvested, as described by Broekaert *et al.* (1990). Five-week-old *pad3-1* mutant plants ( $n=15$ ) on which *P. rapae* had been allowed to feed for 24 hr were challenge-inoculated with *A. brassicicola* by applying 3- $\mu$ L droplets of 10 mM MgSO<sub>4</sub>, containing  $5 \times 10^5$  spores per mL, onto three *P. rapae*-damaged leaves. As a negative control, leaves from untreated plants were inoculated in a similar manner. As a positive control, *pad3-1* plants were pre-treated with MeJA by dipping the leaves in a solution containing 0.1 mM MeJA (Serva, Brunschwig Chemie B.V., Amsterdam, the Netherlands), 24 hr before challenge inoculation. Inoculated plants were kept at 100% relative humidity. At 6 days after challenge, disease severity was determined. Disease ratings were expressed on the basis of intensity of symptoms and lesion size: I, no visible disease symptoms; II, non-spreading lesion; III, spreading lesion without chlorosis; IV, spreading lesion surrounded by chlorotic halo; V, spreading lesion with extensive tissue maceration and sporulation the pathogen.

***Xanthomonas campestris* pv. *armoraciae* and *Pseudomonas syringae* pv. *tomato* bioassays**

Bioassays with the bacterial pathogens *X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato* DC3000 were performed as described by Ton *et al.* (2002) and Pieterse *et al.* (1998). Briefly, rifampicin-resistant *X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato* DC3000 were cultured at 28°C in liquid 0.8% Nutrient Broth medium (Difco, Detroit) and King's medium B (King *et al.*, 1954), respectively. After overnight incubation, bacterial cells were collected by centrifugation and resuspended in 10 mM MgSO<sub>4</sub> containing 0.015% (v/v) Silwet L-77, to a final density of 10<sup>8</sup> and 5x10<sup>6</sup> CFU.mL<sup>-1</sup>, respectively. Five-week-old Arabidopsis plants on which *P. rapae* had been allowed to feed for 24 hr were challenge inoculated by dipping the leaves in the bacterial suspension. Challenge inoculations were performed immediately after removal of the caterpillars, or 3 days later. Three days after challenge inoculation, the percentage of leaves with symptoms was determined per plant ( $n=20$  to 25). Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased. For each plant, caterpillar-damaged leaves (local effects) and undamaged leaves (systemic effects) were scored separately. Mechanically damaged and regurgitant-treated plants were challenged in a similar manner.

Growth of *X. campestris* pv. *armoraciae* and *P. syringae* pv. *tomato* was determined by collecting replicate leaf samples from 10 pools of 3 plants per treatment immediately after challenge inoculation and 3 days later. Leaf samples were weighed, rinsed in water, and homogenized in 10 mM MgSO<sub>4</sub>. Subsequently, dilutions were plated on selective Nutrient Broth or King's medium B supplemented with 100 mg.L<sup>-1</sup> cycloheximide and 50 mg.L<sup>-1</sup> rifampicin. After incubation at 28°C for 2 days, the number of rifampicin-resistant CFU per gram of infected leaf tissue was determined, and bacterial growth over the 3-day time interval was calculated.

**TCV bioassays**

Bioassays with TCV were performed as described previously (Ton *et al.*, 2002). TCV inoculum was produced by *in vitro* transcription from plasmid pT7TCV66 (Oh *et al.*, 1995) and adjusted to a concentration of 0.1 µg of RNA per µL. Five-week-old Arabidopsis Di-0 plants ( $n=15$ ) were challenge inoculated by applying 3-µL droplets of TCV RNA (0.1 µg. µL<sup>-1</sup>) in bentonite buffer (0.05 M glycine, 0.03 M K<sub>2</sub>HPO<sub>4</sub>, 0.02 g of bentonite per mL) on three damaged and three undamaged leaves per plant. On mock-induced plants, six undamaged leaves were inoculated with TCV. Droplets were rubbed across the leaf surface with a glass rod, and the inoculated leaves were marked. Five days after challenge, the number and diameter of the lesions were determined under

a dissection microscope, and viral RNA accumulation was assessed by RNA blot analysis, as described below.

### **Chemical treatments**

Treatments with SA, MeJA and the ET precursor ACC were performed by dipping the leaves in a solution of 0.015% (v/v) Silwet L77, containing either 1 mM SA, 0.1 mM MeJA, 0.1 mM ACC, or a combination of 1 mM SA and MeJA (0.01, 0.1, or 1 mM) or ACC (0.001, 0.01, or 0.1 mM). Control treatments were dipped in a solution containing 0.015% (v/v) Silwet L77.

### **RNA extraction and RNA blot analysis**

Total RNA was extracted as described previously (Van Wees *et al.*, 1999). For RNA blot analysis, 10 µg RNA was denatured using glyoxal and DMSO (Sambrook *et al.*, 1989), electrophoretically separated on 1.5% agarose gel, and blotted onto Hybond-N<sup>+</sup> membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. RNA blots were hybridized with gene-specific probes for *PR-1* and *PDF1.2*, as described previously (Van Wees *et al.*, 1999). To check for equal loading, rRNA bands were stained with ethidium bromide or the blots were stripped and hybridized with a probe for either 18S ribosomal RNA or  $\beta$ -tubulin (*TUB*). The AGI numbers for the genes studied are At2g14610 (*PR-1*), At5g44420 (*PDF1.2*), and At5g44340 (*TUB*). Probes for 18S rRNA and TCV were derived from Arabidopsis cDNA clones, as described (Ton *et al.*, 2002; Verhagen *et al.*, 2004).

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# **Herbivore-induced suppression of host defense gene expression in *Arabidopsis* is regulated by the transcription factor AtMYC2**

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**Keywords:**

Insect herbivores, *Pieris rapae*, insect regurgitant, host defense gene expression, cross-talk, AtMYC2

## Abstract

Plants have to cope with a variety of biotic and abiotic stresses, including mechanical wounding, insect herbivory, and pathogen attack. To minimize damage caused by pathogen or insect attack, plants have evolved sophisticated defense mechanisms. In turn, specialized attackers exhibit ways to circumvent recognition by the plant or have the ability to interfere with host defense responses to create an optimal environment for themselves. Caterpillars of the cabbage white butterfly, *Pieris rapae*, have adapted to feed on crucifers. In *Arabidopsis*, wounding inflicted by feeding *P. rapae* larvae did not induce the expression of the jasmonate (JA)-responsive marker gene *PDF1.2*, while similar wounding caused by mechanical damage strongly activated the *PDF1.2* gene. Application of *P. rapae* regurgitant onto the mechanically wounded sites mimicked the *P. rapae*-mediated suppression of *PDF1.2*, suggesting that elicitors in the caterpillar's regurgitant actively suppress this host defense response. Conversely, other JA-responsive genes, such as *VSP2* and *LOX2*, showed increased expression upon caterpillar feeding and were not induced by wounding, indicating that upon *P. rapae* feeding, different JA-regulated host defenses are activated or suppressed. To investigate the molecular mechanism by which *P. rapae* feeding suppresses *PDF1.2* expression, we studied the role of salicylic acid (SA) and abscisic acid (ABA), both of which have been implicated in antagonizing the JA-induced expression of *PDF1.2*. Suppression of *PDF1.2* by *P. rapae* was unchanged in the SA-signaling mutant *npr1-1*, suggesting that SA is not involved in the herbivore-induced suppression of *PDF1.2*. However, the ABA biosynthesis mutant *aba2-1* showed a significantly increased *PDF1.2* expression upon feeding by *P. rapae*. Previously, ABA was shown to be an important regulator of *AtMYC2*, a transcription factor that activates a specific set of JA-responsive genes (e.g. *VSP2* and *LOX2*), while it suppresses other JA-responsive genes (e.g. *PDF1.2*). The *AtMYC2* gene was up-regulated in response to *P. rapae* feeding, but not as a result of mechanical damage. Like *aba2-1*, the *AtMYC2* mutant *jin1-2* was also impaired in *P. rapae*-induced suppression of *PDF1.2* and showed high levels of *PDF1.2* expression upon *P. rapae* feeding. Suppression of other wound-responsive genes with a similar *P. rapae*-suppressed expression pattern in wild-type Col-0 plants, e.g. *ETHYLENE-RESPONSE FACTOR*, showed a strong *P. rapae*-mediated expression pattern in *jin1-2*. Taken together, our results indicate that activation of *AtMYC2* is important for *P. rapae*-mediated suppression of a specific branch of the JA-dependent host defense response.

## Introduction

Plants possess different inducible defense mechanisms to cope with attack by microbial pathogens and herbivorous insects. To understand how plants integrate pathogen- and insect-induced signals into specific defense responses, we previously monitored the dynamics of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signaling in Arabidopsis after attack by a set of microbial pathogens and herbivorous insects with different modes of attack (De Vos *et al.*, 2005). We investigated the response of Arabidopsis to the well-characterized microbial pathogens *Pseudomonas syringae* pv. *tomato* and *Alternaria brassicicola*, and the herbivorous insects *Pieris rapae*, *Myzus persicae*, and *Frankliniella occidentalis*. The production of the signal molecules SA, JA, and ET was monitored during these five interactions and related to global gene expression profiles using Affymetrix ATH1 whole-genome GeneChips. We showed that JA played an important role in the differential regulation of a large proportion of the activated/repressed genes in four out of the five Arabidopsis-attacker combinations tested. Nevertheless, the vast majority of the JA-responsive changes in gene expression were specific for individual plant-attacker combinations (De Vos *et al.*, 2005). Hence, JA plays an important role in the primary response to these pathogens and insects, but additional layers of regulation shape the final outcome of the defense reaction (De Vos *et al.*, 2005).

Many studies have indicated that JA and its derivatives are among the most important regulators of induced resistance against herbivore attack. A classic example is the observation that following attack by larvae of *Manduca sexta*, tomato leaves accumulate JA, resulting in the activation of genes encoding proteinase inhibitor proteins that inhibit digestive serine proteinases of herbivorous insects and reduce further insect feeding (Farmer and Ryan, 1992; Howe, 2005). Likewise, genetic evidence demonstrates that JA plays an important role in induced defense against different types of herbivores in Arabidopsis (Ellis *et al.*, 2002; McConn *et al.*, 1997; Reymond *et al.*, 2004; Stintzi *et al.*, 2001; Stotz *et al.*, 2002; Van Poecke and Dicke, 2004). Mechanical damage and feeding by *P. rapae* caterpillars on Arabidopsis leads to an increased production of JA and the expression of JA-responsive genes (De Vos *et al.*, 2005; Reymond *et al.*, 2000; 2004). Although JA levels increase not only upon caterpillar damage, but also in reaction to wounding, the response to these stimuli is not identical. Reymond *et al.* (2004) observed a remarkably small overlap in transcript profiles between mechanical damage and feeding by larvae of the cabbage white butterfly (*P. rapae*), suggesting a role for additional factors in the modulation of the response to caterpillar feeding.

How can these differences between mechanical damage and herbivore feeding be explained? First of all, artificially wounded leaf tissues do not produce the same blends of volatiles as leaf tissues that have been injured by feeding herbivores (Mattiacci *et al.*, 1995; Van Poecke and Dicke, 2002). Using a mechanical caterpillar, named MecWorm, Mithöfer *et al.* (2005) demonstrated that computerized continuous damage resembles the insect's feeding process much better, leading to the production of a volatile blend that is more similar than wounding at a single time point. Evidently, the dynamics of wounding inflicted by feeding herbivores modify the nature of the induced plant defense response to a large extent. Secondly, herbivore-derived elicitors that are released upon feeding can influence the wound response. Application of regurgitant from feeding herbivores to mechanically damaged sites has been demonstrated to mimic specific herbivore-induced defense responses. For instance, cabbage leaves that are artificially damaged and subsequently treated with regurgitant of *Pieris brassicae* larvae, release a volatile blend similar to that of herbivore-damaged plants (Mattiacci *et al.*, 1995). Insect-derived compounds, such as the enzyme  $\beta$ -glucosidase and fatty acid-amino acid conjugates, e.g. volicitin, have been identified as potent elicitors of volatile production in different plant-herbivore interactions (Halitschke *et al.*, 2001; Mattiacci *et al.*, 1995; Turlings *et al.*, 2000). Besides insect-derived elicitors, caterpillar regurgitant also contains high levels of plant-derived molecules, including oxylipins, such as JA, the jasmonate precursor 12-oxo-phytodienoic acid (OPDA), and dinor oxo-phytodienoic acid (dnOPDA) (Reymond *et al.*, 2004), which have been shown to play a critical role in herbivore resistance in *Arabidopsis* (Stintzi *et al.*, 2001).

As specialist herbivores can develop successfully on one or a few related plant species, it has been hypothesized that continuing co-evolution has provided them with mechanisms to avoid recognition by the host plant (Schoonhoven *et al.*, 2005). Moreover, this evolutionary arms race between plant and attacker may result in detoxification mechanisms in the insect. For instance, *Arabidopsis* deploys a chemical defense system, called 'the mustard oil bomb', against herbivorous attackers (Rask *et al.*, 2000). In this system, glucosinolates are cleaved by myrosinase enzymes, releasing toxic compounds and repellent volatiles that are effective against many generalist herbivores (Wittstock *et al.*, 2003). Specialist *P. rapae* larvae are not affected by these glucosinolate breakdown products. Recently, Wittstock *et al.* (2004) demonstrated that a larval gut protein from *P. rapae* prevents formation of toxic breakdown products by redirecting glucosinolate hydrolysis toward nitrile formation. Thus insect-derived compounds, such as salivary proteins, can modulate or interfere with expression of host defenses.

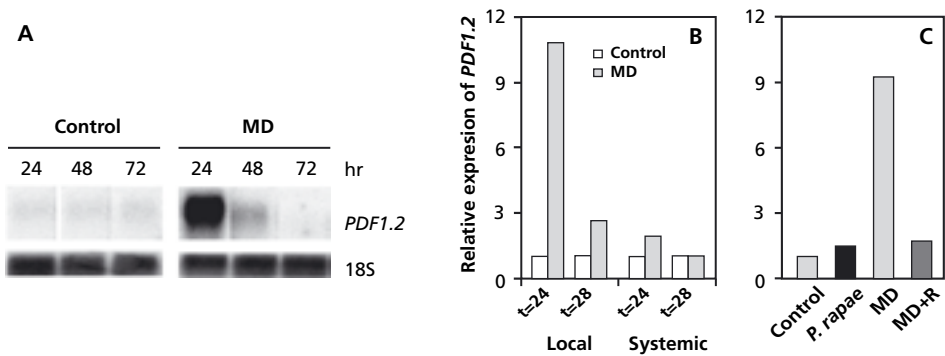
Previously, we tested the effectiveness of *P. rapae*-induced resistance against different microbial pathogens (Chapter 3). *P. rapae* feeding induces JA biosynthesis in damaged tissue (De Vos *et al.*, 2005). Although the necrotrophic fungus *A. brassicicola* is sensitive to JA-dependent defenses, *P. rapae* feeding was not effective in inducing resistance against this pathogen. *PLANT DEFENSIN1.2* (*PDF1.2*), a JA-responsive marker gene for resistance against *A. brassicicola* (Penninckx *et al.*, 1996; 2003), was suppressed by elicitors in the regurgitant of *P. rapae* (Chapter 3), suggesting that this herbivore actively suppresses the JA-dependent defense response that is associated with resistance against this necrotrophic pathogen. Evidence is accumulating that different defense pathways cross-communicate (Dicke and Van Poecke, 2002; Felton and Korth, 2000; Feys and Parker, 2000; Kunkel and Brooks, 2002; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998; Rojo *et al.*, 2003), providing the plant with a powerful regulatory potential to fine-tune its defense response to the attacker encountered. JA-responsive gene expression, in particular the marker gene *PDF1.2*, has been demonstrated to be modulated by cross-talk between signal transduction pathways. For instance, SA-mediated inhibition of JA-responsive *PDF1.2* gene expression has been studied in substantial detail (Spoel *et al.*, 2003). In addition, the plant hormone abscisic acid (ABA) has been shown to be a potent inhibitor of *PDF1.2* expression and some, but not all, other JA-responsive genes, through the action of the transcription factor protein AtMYC2 (Anderson *et al.*, 2004). Using a pharmacological approach, Lorenzo *et al.* (2004) demonstrated that the transcription factors AtMYC2 and ERF1 antagonistically regulate two sets of JA-responsive genes: AtMYC2 represses JA-responsive genes that are involved in defense against pathogens (e.g. *PDF1.2*), whereas ERF1 acts as a positive regulator in this respect (Lorenzo *et al.*, 2004).

Here, we investigated the mechanism by which *P. rapae* feeding suppresses the activation of *PDF1.2* gene expression in Arabidopsis. We demonstrate that *P. rapae*-mediated suppression of *PDF1.2* functions independently of SA, but is regulated by ABA and the transcription factor AtMYC2.

## Results

### Wound-induced *PDF1.2* is suppressed by *P. rapae* regurgitant

Plant responses upon wounding are induced to protect damaged leaves against water loss and attack by opportunistic pathogens. These responses are primarily regulated by the plant hormone JA and, in part, resemble the response



**Figure 1.** *P. rapae*-mediated suppression of wound-induced *PDF1.2* expression

**A.** Northern blot analysis of *PDF1.2* gene expression in control and mechanically damaged leaves, at 24, 48, and 72 hr after wounding. Equal loading of RNA samples was checked using a probe for 18S rRNA. **B.** Q-RT-PCR analysis of local and systemic expression of *PDF1.2* in control and mechanically damaged Col-0 plants. *PDF1.2* expression levels are given relative to the *PDF1.2* mRNA levels in untreated control plants (set at 1). **C.** Q-RT-PCR analysis of relative *PDF1.2* mRNA levels in Col-0 plants, 24 hr after infestation with first-instar larvae of *P. rapae*, mechanical damage (MD), or mechanical damage followed by treatment with caterpillar regurgitant (MD+R). *PDF1.2* expression levels are given relative to the *PDF1.2* mRNA levels in untreated control plants (set at 1).

that is induced upon insect feeding (Howe, 2005). Although mechanical damage has been extensively studied in order to understand plant defense responses to herbivore feeding, wounding alone does not fully mimic these responses (Halitschke *et al.*, 2001; Mattiacci *et al.*, 1995; Reymond *et al.*, 2000; Turlings *et al.*, 2000; Van Poecke *et al.*, 2001). Insect-derived factors, such as elicitors present in regurgitant, can modulate host gene expression. In particular, specialist caterpillars appear able to suppress host responses by interfering with defense signaling pathways. Figure 1A shows that mechanical wounding of Arabidopsis leaves induced a transient expression of the JA-responsive marker gene *PDF1.2* in the wounded leaves, with a peak at 24 hr after wounding. Wound-induced expression of *PDF1.2* could not be detected in systemic tissues (Fig. 1B), indicating that the effect is local. Arabidopsis leaves that were infested for 24 hr by first-instar larvae of *P. rapae* did not show this increase in *PDF1.2* gene expression (Fig. 1C), even though they were damaged to a similar extent as mechanically wounded leaves and JA levels were increased similarly in these tissues (data not shown). Application of *P. rapae* regurgitant onto the damaged sites resulted in a suppression of the wound-induced *PDF1.2* expression (Fig. 1C). These results suggest that *P. rapae*-derived elicitors are involved in the suppression of *PDF1.2* gene expression that is activated upon wounding.

### ***P. rapae* feeding suppresses a specific branch of the JA response**

To investigate whether, besides *PDF1.2*, other JA-responsive genes are suppressed similarly by elicitors in the *P. rapae* regurgitant, we examined the

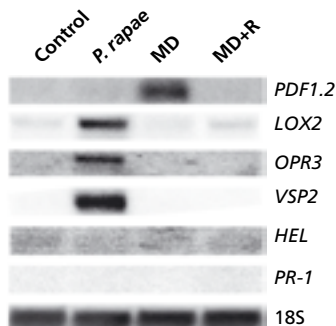
expression of the well-characterized JA-responsive genes *LIPOXYGENASE2* (*LOX2*) and *12-OXOPHYTODIENOATE REDUCTASE3* (*OPR3*), which encode key enzymes in the JA biosynthetic pathway (Bell *et al.*, 1995 and Schaller *et al.*, 2000, respectively), and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*). Arabidopsis plants were infested with three freshly hatched larvae of *P. rapae*. Other plants were mechanically damaged and treated, or not, with caterpillar regurgitant. Figure 2 shows that in contrast to *PDF1.2*, the expression of *LOX2*, *OPR3*, and *VSP2* was strongly induced by *P. rapae* feeding, indicating that the expression of these JA-responsive genes is not suppressed during the Arabidopsis-*P. rapae* interaction. Unlike *PDF1.2*, these three genes were not activated in response to wounding, indicating that *P. rapae* feeding and wounding differentially activate specific JA-responsive genes.

### ***P. rapae*-mediated suppression of *PDF1.2* is NPR1-independent**

Previously, the regulatory protein NPR1 was shown to play a crucial role in SA-mediated inhibition of JA-induced expression of *PDF1.2* (Spoel *et al.*, 2003). To investigate whether *P. rapae*-induced suppression of *PDF1.2* is dependent on NPR1, we studied *PDF1.2* mRNA levels in *npr1-1* mutant plants infested with freshly hatched *P. rapae* larvae. Figure 3A shows that, like wild-type Col-0 plants, mutant *npr1-1* plants did not accumulate *PDF1.2* transcripts after infestation with *P. rapae*. These results indicate that suppression of *PDF1.2* by *P. rapae* is independent of the regulatory protein NPR1 and, therefore, does not involve SA-mediated inhibition of JA-responsive gene expression. These results are in good agreement with previous findings (De Vos *et al.*, 2005) that *P. rapae* induces neither the production of SA, nor the expression of SA-responsive *PR-1* (Fig. 2).

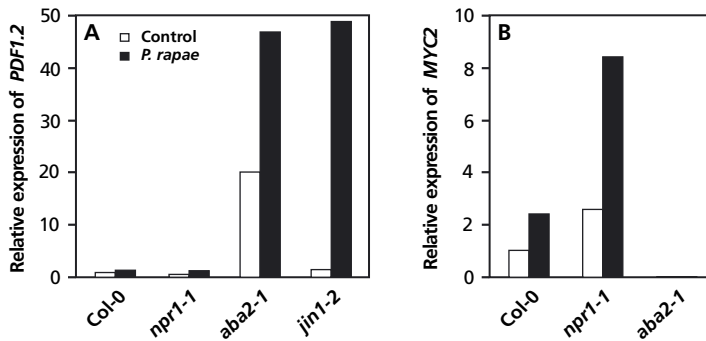
### **Role for ABA in herbivore-mediated suppression of *PDF1.2***

Water loss upon wounding or herbivore feeding has been suggested to cause dehydration stress, resulting in an enhanced level of the plant hormone



**Figure 2.** Differential expression of defense-related marker genes in response to *P. rapae* feeding, mechanical damage, and regurgitate treatment.

Northern blot analysis of the JA-responsive genes *PDF1.2*, *LOX2*, and *OPR3*, (the ET-responsive gene *HEL*), and the SA-responsive gene *PR-1* 24 hr after infestation by first-instar larvae of *P. rapae*, mechanical damage (MD), or mechanical damage followed by treatment with caterpillar regurgitant (MD+R). Equal loading of RNA samples was checked using a probe for 18S rRNA.



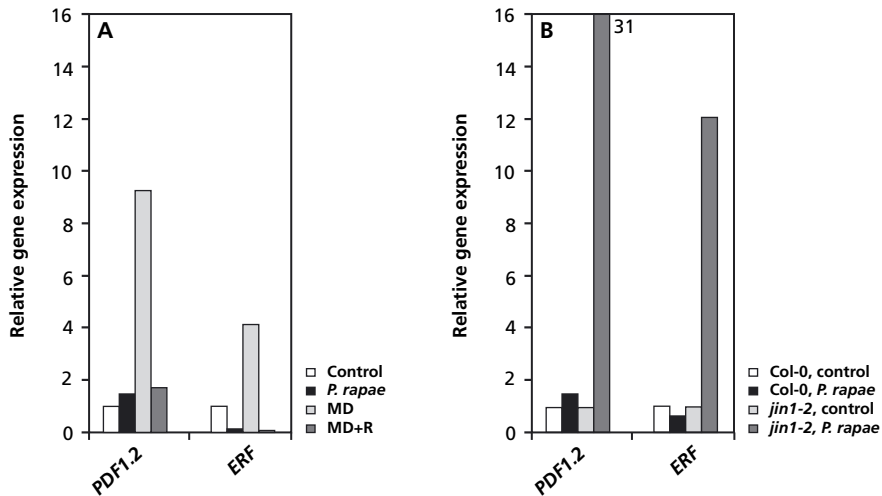
**Figure 3.** Expression patterns of *PDF1.2* and *AtMYC2* in wild-type Col-0, and mutant *npr1-1*, *aba2-1*, and *jin1-2* plants 24 hr upon *P. rapae* feeding.

Q-RT-PCR analysis of *PDF1.2* (A) and *AtMYC2* (B) transcript levels in untreated and *P. rapae*-infested plants of the wild-type Col-0, the SA-response mutant *npr1-1*, the ABA biosynthesis mutant *aba2-1*, and the *AtMYC2*-defective mutant *jin1-2*. *PDF1.2* and *AtMYC2* mRNA levels are given relative to that in uninfested (Control) Col-0 plants, which was set at 1.

abscisic acid (ABA) and associated induction of ABA-responsive genes (Denekamp *et al.*, 2003; Reymond *et al.*, 2000). Recently, Anderson *et al.* (2005) showed that ABA is a powerful modulator of JA action. In their studies, JA-responsive *PDF1.2* expression was strongly suppressed upon exogenous application of ABA. Furthermore, ABA biosynthesis mutants, e.g. *aba2-1*, showed high constitutive expression of *PDF1.2*. To study the role of ABA in *P. rapae*-mediated suppression of *PDF1.2*, we determined *PDF1.2* transcript levels in the ABA biosynthesis mutant *aba2-1*. In contrast to wild-type Col-0 plants, *aba2-1* plants exhibited high constitutive expression of *PDF1.2*, confirming previous findings (Anderson *et al.*, 2004). Moreover, upon *P. rapae* feeding the steady-state *PDF1.2* transcript level was more than doubled (Fig. 3A), suggesting that in wild-type plants ABA is involved in *P. rapae*-mediated suppression of *PDF1.2*.

### **AtMYC2 is required for herbivore-mediated suppression of *PDF1.2***

Using a pharmacological approach, Anderson *et al.* (2004) and Lorenzo *et al.* (2004) provided evidence that suppression of *PDF1.2* by ABA is regulated by the transcription factor AtMYC2. AtMYC2 differentially regulates two distinct groups of JA-responsive genes. One group is repressed by AtMYC2 and includes genes involved in defense against pathogens (e.g. *PDF1.2*). A second group is activated by AtMYC2 and includes JA-responsive genes such as *VSP2*. To investigate whether *P. rapae*-mediated suppression of *PDF1.2* is also regulated by AtMYC2, we infested mutant *jin1-2* plants with freshly hatched larvae. Mutant *jin1-2* plants contain a point mutation in the coding sequence of the *AtMYC2* gene, which leads to an early stop-codon and an



**Figure 4.** Expression patterns of *PDF1.2* and *ERF* in Col-0 and *jin1-2*, in response to *P. rapae* feeding, wounding, and regurgitant treatment.

(A) Q-RT-PCR analysis of *PDF1.2* and *ERF* transcript levels in untreated, *P. rapae*-infested, mechanically damaged (MD), and regurgitant treated (MD+R). (B) *PDF1.2* and *ERF* expression in untreated and *P. rapae*-treated wild-type Col-0 and mutant *jin1-2* plants. *PDF1.2* and *ERF* mRNA levels are given relative to that in the uninfested control, which was set at 1. Leaf tissues were harvested 24 hr after treatment.

ineffective protein (Lorenzo *et al.*, 2004). Figure 3A shows that *jin1-2* plants, unlike *aba2-1* plants, do not express *PDF1.2* constitutively, but unlike Col-0 and *npr1-1* plants, accumulate high levels of *PDF1.2* mRNA in response to *P. rapae* feeding. Thus, in wild-type plants *AtMYC2* is required for *P. rapae*-mediated suppression of *PDF1.2*. To verify the role of ABA in the activation of *AtMYC2*, we analyzed the expression of *AtMYC2* in Col-0, *npr1-1*, and *aba2-1* plants. *AtMYC2* transcript levels were significantly increased in both Col-0 and *npr1-1* upon feeding by *P. rapae*, which correlates with the suppression of *PDF1.2* (Fig. 3B). In addition, both steady-state and *P. rapae*-induced levels of *AtMYC2* mRNA were decreased to undetectable levels in *aba2-1*, indicating that ABA is required for *P. rapae*-induced expression of *AtMYC2*. Together, these data indicate that *P. rapae* feeding induces *AtMYC2* gene expression in an ABA-dependent manner, resulting in the suppression of *PDF1.2* gene expression.

#### ***P. rapae*-mediated suppression of wound-induced host genes**

To identify wound-induced genes that, like *PDF1.2*, are suppressed upon *P. rapae* feeding, we made use of available whole-genome expression profiles of *P. rapae*-infested Arabidopsis plants (De Vos *et al.*, 2005), and that of Arabidopsis

plants that were mechanically damaged (Harter laboratory, Cologne University, Germany; see acknowledgments). Leaf tissue used for the Affymetrix ATH1 GeneChip analysis was from 18-day-old *Arabidopsis* Col-0 plants that were damaged by puncturing the leaves. Subsequently, shoot tissue was harvested at several time points after wounding for RNA extraction. Expression data from approximately 23,000 genes was assessed with ATH1 GeneChip technology from Affymetrix. For our purpose, we selected genes that showed an at least 3-fold up-regulation upon mechanical damage at both 12 and 24 hr after wounding. In total, 273 genes matched these selection criteria (Supplementary Table 1; [http://www.bio.uu.nl/~fytopath/GeneChip\\_data.htm](http://www.bio.uu.nl/~fytopath/GeneChip_data.htm)). Out of these 273 genes, we selected all genes that were either unchanged (<1,25-fold up in comparison to uninfested plants) or down-regulated at 12 and 24 hr after *P. rapae* feeding. This selection yielded 63 wound-inducible genes that were suppressed upon *P. rapae* feeding (Supplementary Table 1). As an illustration, *PDF1.2* (At5g44420) expression was up-regulated 7.1- and 21.1-fold at 12 and 24 hr after wounding, respectively, but not upon *P. rapae* feeding (-1.4 at both 12 and 24 hr after feeding), in agreement with previous findings (Fig. 1; Chapter 3).

To validate the GeneChip data and to investigate whether factors in the caterpillar regurgitant are involved in the *P. rapae*-mediated suppression, we selected a gene encoding an AP2-domain transcription factor from the ETHYLENE-RESPONSE FACTOR family (At1g06160) and studied its expression in response to herbivore feeding, wounding and regurgitate treatment in an independent experiment. We chose this gene because i) this wound-inducible *ERF* gene was among the strongest down-regulated genes in *P. rapae*-infested plants, and ii) it is homologous to *ERF1*, which has been demonstrated to be an important positive regulator of *PDF1.2* expression. Figure 4A shows that the *ERF* gene was strongly induced upon mechanical damage, but completely suppressed upon *P. rapae* feeding, confirming the GeneChip data. Moreover, the wound-induced expression levels of the *ERF* gene were strongly suppressed upon treatment of the wounded sites with regurgitant from *P. rapae*. These results indicate that, like *PDF1.2*, also the *ERF* gene is suppressed by elicitors in the regurgitant of *P. rapae* (Fig. 4a).

To elucidate the role of AtMYC2 in the *P. rapae*-mediated suppression of the *ERF* gene, we analyzed *ERF* and *PDF1.2* mRNA levels in infested and uninfested Col-0 and *jln1-2* plants. Figure 4B shows the relative expression of the genes upon *P. rapae* infestation compared to untreated control plants. Both *ERF* and *PDF1.2* are strongly induced in *jln1-2* plants in response to herbivore feeding, while in wild-type Col-0 plants the expression of these genes remained unchanged (Fig. 4B). It can, thus, be concluded that AtMYC2 plays an important role in the *P. rapae*-mediated suppression of these host defense genes.

## Discussion

As a result of the evolutionary arms race between plants and their attackers, plants have evolved sophisticated defense mechanisms, while effective attackers developed ways to circumvent or overcome these responses. In contrast to mechanical damage, wounding of Arabidopsis leaves by feeding larvae of the specialist herbivore *P. rapae* did not induce the expression of wound-inducible genes such as *PDF1.2*. Application of regurgitant of *P. rapae* to mechanically damaged sites strongly suppressed the wound-induced expression of *PDF1.2*, suggesting that *P. rapae* actively suppresses host defenses that were induced as a result of wounding. Here, we investigated the underlying mechanism of *P. rapae*-mediated suppression of wound-induced *PDF1.2* expression.

Expression of *PDF1.2* is known to be regulated by the concomitant action of the signaling compounds JA and ET (Pennickx *et al.*, 1996). Therefore, suppression of wound-induced *PDF1.2* mRNA levels by *P. rapae* feeding could be explained if herbivore-damaged plants produced far less JA and/or ET than wounded plants. We have previously shown that *P. rapae* feeding enhances the production of both JA and ET in Arabidopsis (De Vos *et al.*, 2005). Reymond *et al.* (2000; 2004) showed that plants under attack by *P. rapae* or artificially wounded, increased JA levels in their leaves to a similar extent. Moreover, we observed that *P. rapae*- and mechanically damaged plants produced similar levels of ET (data not shown), ruling out a role for decreased JA or ET levels in the suppression of wound-induced *PDF1.2*.

SA is a powerful suppressor of JA-responsive genes such as *PDF1.2*, *VSP2*, and *LOX2* (Spoel *et al.*, 2003). In our study, *P. rapae* feeding only suppressed the expression of *PDF1.2*, and activated the JA-responsive genes *VSP2*, *LOX2*, and *OPR3* (Fig. 2). This suggests that *P. rapae* induces a specific subset of JA-responsive genes (e.g. *VSP2*, *LOX2*, and *OPR3*), while suppressing another subset (e.g. *PDF1.2*). This observation makes a role for SA-mediated cross-talk unlikely, since SA has been shown to suppress the expression of *VSP2* and *LOX2* (Spoel *et al.*, 2003). We showed here that *P. rapae*-mediated suppression of *PDF1.2* is not affected in the SA-signaling mutant *npr1-1* (Fig. 3A). This result, together with the observation that feeding of *P. rapae* larvae on Arabidopsis is not associated with enhanced levels of SA and *PR-1* mRNA (Fig. 2; De Vos *et al.*, 2005), indicates that SA does not play a role in the suppression of wound-induced *PDF1.2* gene expression.

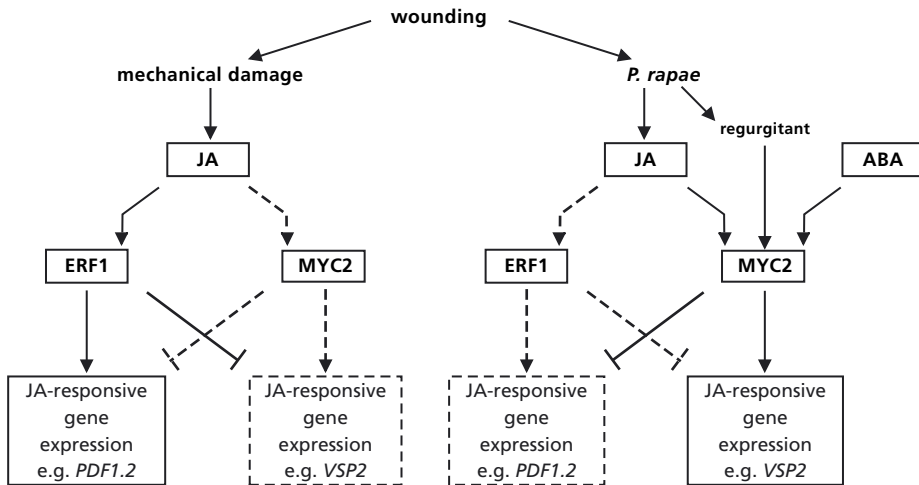
Besides SA, ABA has been demonstrated to suppress JA-responsive gene expression. Anderson *et al.* (2004) demonstrated that exogenous application of ABA suppressed both basal and JA-activated transcription of *PDF1.2*. Moreover, ABA deficiency as conditioned by mutations in the ABA biosynthesis genes *ABA1* or *ABA2*, resulted in upregulation of both basal and JA-induced

transcription of *PDF1.2*, indicating that ABA plays an important role in the suppression of *PDF1.2*. Here, we demonstrated that the ABA biosynthetic mutant *aba2-1* is blocked in its ability to suppress *PDF1.2* expression, leading to high *PDF1.2* transcript levels upon feeding by *P. rapae* (Fig. 3A). These results indicate that ABA is required for *P. rapae*-mediated suppression of *PDF1.2*.

The basic helix-loop-helix-leucine zipper transcription factor AtMYC2, is a positive regulator of ABA signaling (Abe *et al.*, 2003; Anderson *et al.*, 2004), and was previously shown to be essential for discriminating between different JA-regulated defense responses in Arabidopsis. On the one hand, AtMYC2 positively regulated JA-induced expression of a subset of JA-responsive genes, such as *VSP2* and *LOX2*. On the other hand, the AtMYC2 was found to suppress another subset of JA-responsive genes, including *PDF1.2* (Lorenzo *et al.*, 2004). In our study, we showed that expression of *AtMYC2* gene is activated upon *P. rapae* feeding in an ABA-dependent manner (Fig. 3B), and that AtMYC2 is required for the *P. rapae*-mediated suppression of *PDF1.2* as well as a wounding- and JA-responsive *ERF* gene (Fig. 3A and 4B). Because regurgitant mimicks the suppression of these host defense genes, elicitors in the regurgitant of *P. rapae* appear to activate ABA-dependent suppression of *PDF1.2* through AtMYC2. Analysis of whole-genome microarray data revealed 63 wound-inducible genes that are suppressed upon *P. rapae* feeding, suggesting that *P. rapae* antagonizes a large set of host genes that are normally activated in response to wounding.

Figure 5 provides a working model of how Arabidopsis plants could integrate mechanical damage- and *P. rapae*-induced signals into specific JA-responsive host defenses. Wounding, such as caused by mechanical damage, induces JA production, leading to the ERF-dependent expression of a subset of JA-responsive genes (e.g. *PDF1.2*). However, in combination with elicitors in the regurgitant of *P. rapae*, the transcription factor gene *AtMYC2* is activated in an ABA-dependent manner. This results in the suppression of the ERF1-controlled subset of JA-responsive genes, and the up-regulation of another set of JA-responsive genes (e.g. *VSP2*).

It appears clear that specialized attackers, such as *P. rapae*, have found ways to manipulate host plant defenses by interfering with the plant's defense signaling pathways. Redirecting the plant's defense responses by interfering with cross-talk mechanisms would be an evolutionary advantage for the attacker. This phenomenon is difficult to counter by the host, and may be a long lasting strategy for successful invasion of the host plant. Future research will be focused on the relation between herbivore-induced suppression of host defenses and herbivore performance.



**Figure 5.** Schematic model for the role of AtMYC2 in *P. rapae*-mediated suppression of JA-responsive host defense genes. Solid lines indicate induced activity upon a particular stimulus, while the dotted lines show the suppressed signal transduction pathways. Partially adapted from Lorenzo *et al.*, 2004, Anderson *et al.*, 2004, and Lorenzo and Solano, 2005.

## Materials and methods

### Cultivation of plants

Seeds of *Arabidopsis thaliana* accession Col-0 and the Col-0 mutants *npr1-1* (Cao *et al.*, 1994), *aba2-1* (Koornneef *et al.*, 1982), and *jln1-2* (Lorenzo *et al.*, 2004) were sown in quartz sand. Two-week-old seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min. Plants were cultivated in a growth chamber with a 8-hr day (200  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  at 24°C) and 16-hr night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing 10  $\mu\text{M}$  Sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

### Wounding, herbivore feeding, and regurgitant treatment

The response to wounding was assessed by mechanically damaging the leaves of 5-week-old Arabidopsis plants ( $n=10$ ). To this end, 3 small holes (1 mm diameter) were punctured in each of 6 leaves per plant using a sterile needle. Wounded local and untreated (systemic) leaf material was harvested at 24, 48, and 72 hr after damage.

To investigate in how far wounding resembles *P. rapae* feeding, 5-week-old Arabidopsis plants were infested with 3 first-instar (L1) larvae that had freshly hatched on plants of Brussels sprout (*Brassica oleracea gemmifera* cv. Cyrus), as previously described (De Vos *et al.*, 2005). Larvae were allowed to

feed for 24 hr, after which the leaves were harvested. To study the effect of *P. rapae* regurgitant, 1  $\mu$ L of freshly collected regurgitant from L4/L5-instar *P. rapae* larvae was divided over the 3 punctured holes of each mechanically damaged leaf (Mattiacci *et al.*, 1995). Damaged leaf material was harvested 24 hr after the start of the induction treatments. These experiments were performed twice with similar results.

### RNA extraction and northern blotting

Total RNA was extracted as described previously (De Vos *et al.*, 2005). For northern blot analysis, 10  $\mu$ g RNA was denatured using glyoxal and DMSO (Sambrook *et al.*, 1989), electrophoretically separated on 1.5% agarose gel, and blotted onto Hybond-N<sup>+</sup> membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. Northern blots were hybridized with gene-specific probes for *PDF1.2*, *LOX2*, *OPR3*, *VSP2*, *HEL*, and *PR-1*, as described previously (Van Wees *et al.*, 1999). To check for equal loading, the blots were stripped and hybridized with a probe for 18S rRNA. The AGI numbers of the genes studied are At5g44420 (*PDF1.2*), At3g45140 (*LOX2*), At2g06050 (*OPR3*), At5g24770 (*VSP2*), At3g04720 (*HEL*), and At2g14610 (*PR-1*). The probe for 18S was derived from Arabidopsis cDNA clones, as described (Verhagen *et al.*, 2004).

### Quantitative real-time PCR

Q-RT-PCR analysis was performed basically as described previously (Czechowski *et al.*, 2004). Five  $\mu$ g of RNA was digested with Turbo DNA-free<sup>TM</sup> (Ambion, Huntingdon, United Kingdom) according to the manufacturer's instructions. To check for genomic DNA contamination, a PCR with primers designed on intron sequences of *ACT7* (At5g09810; *ACT7*-FOR; 5'-GAC ATG GAA AAG ATA TGG CAT CAC AC-3'; *ACT7*-REV; 5'-AGA TCC TTC CTG ATA TCG ACA TCA C-3') was carried out. Subsequently, DNA-free total RNA was converted into cDNA using oligo-dT<sub>20</sub> primers (Invitrogen, Breda, the Netherlands), 10 mM dNTPs, and SuperScript<sup>TM</sup> III Reverse Transcriptase (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Efficiency of cDNA synthesis was assessed by Q-RT-PCR using primers of the constitutively expressed gene *UBI10* (At4g05320; *UBI10*-FOR; 5' AAA GAG ATA ACA GGA ACG GAA ACA TAG T-3'; *UBI10*-REV; 5'-GGC CTT GTA TAA TCC CTG ATG AAT AAG-3'). Gene-specific primers were designed for *PDF1.2* (At5g44420; FOR 5'-CGA GAA GCC AAG TGG GAC AT-3'; REV 5'-TCC ATG TTT GGC TCC TTC AA-3'), *AtMYC2* (At1g32640; FOR 5'-ATA AAA CCG CCG GAG AAT CAG-3'; REV 5'-GCT GGC TTT CTT CCT CGT TTC-3'), and a gene from the

*ERF* gene family (At1g06160), which showed a similar expression pattern as *PDF1.2* in *P. rapae*-infested and wounded Arabidopsis leaves. The following primers were used to detect At1g06160: FOR 5'-TTC CCC GGA GAA CTC TTC TT-3', REV 5'-GCC TGA TCA TAA GCG AGA GC-3'. Q-RT-PCR analysis was performed in optical 96-well plates with a MyIQ™ Single Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands), using SYBR® Green to monitor dsDNA synthesis. Each reaction contained 1 µL of cDNA, 0.5 µL of each of the two gene-specific primers (10 pmol.µL<sup>-1</sup>), and 10 µL of 2x IQ SYBR® Green Supermix reagent (Bio-Rad, Veenendaal, the Netherlands) in a final volume of 20 µL. The following PCR program was used for all PCR reactions: 95 °C for 3 min; 40 cycles of 95 °C for 30 sec, 59.5 °C for 30 sec, and 72 °C for 30 sec. C<sub>T</sub> (threshold cycle) values were calculated using Optical System Software, version 1.0 for MyIQ™ (Bio-Rad, Veenendaal, the Netherlands). Subsequently, C<sub>T</sub> values were normalized for differences in dsDNA synthesis using the *UBI10* C<sub>T</sub> values. Normalized transcript levels of the genes tested were compared between treatments and the fold change in expression level was calculated.

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**Supplementary table 1:** Selected genes that show an at least 3-fold expression at 12 and 24 hr upon wounding (Harter lab) and that are, like *PDF1.2*, not induced (or suppressed) upon 12 and 24 hr of *P. rapae* attack (De Vos *et al.*, 2005). This supplementary table can be found at [http://www.bio.uu.nl/~fytopath/GeneChip\\_data.htm](http://www.bio.uu.nl/~fytopath/GeneChip_data.htm).



# Role for the AtMYB102 transcription factor in the defense of Arabidopsis against tissue-chewing insects

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## Key words:

*Pieris rapae*, insect resistance, *Arabidopsis thaliana*, R2R3-myb transcription factor, AtMYB102

## Abstract

In Arabidopsis the R2R3-MYB transcription factor family consists of over 100 members and is implicated in many biological processes, such as plant development, metabolism, senescence, and defense. The R2R3-MYB transcription factor gene *AtMYB102* has been shown to respond to salt stress, ABA, JA, and wounding, suggesting that *AtMYB102* plays a role in the response of plants to dehydration after wounding. Here, we studied the role of *AtMYB102* in the response of Arabidopsis to feeding by larvae of the white cabbage butterfly *Pieris rapae*. Arabidopsis reporter lines expressing *GUS* under control of the *AtMYB102* promoter revealed that *AtMYB102* is expressed locally at the feeding sites of herbivore-damaged leaves, but not systemically in uninfested plant parts. Knock-out *AtMYB102* T-DNA insertion mutant plants (*myb102*) allowed a faster development of *P. rapae* caterpillars than wild-type Col-0 plants. Moreover, the number of caterpillars that had developed into pupae within 14 days was significantly higher on *myb102*, indicating that in wild-type plants *AtMYB102* contributes to basal resistance against *P. rapae* feeding. *AtMYB102* over-expressing 35S:*MYB102* plants did not show an enhanced resistance to feeding by *P. rapae* larvae. To analyze the effect of constitutive *MYB102* gene expression, transcript profiling of wild-type and over-expressor 35S:*MYB102* plants was performed. A total of 268 genes was found to be differentially expressed. A relatively large proportion of genes that were up-regulated in the over-expressor appeared to be associated with the cell wall, suggesting that MYB102 plays a role in regulating the capacity for cell wall remodeling.

## Introduction

Plants possess a broad range of defense mechanisms to effectively combat invasion by microbial pathogens or attack by herbivorous insects. These mechanisms include pre-existing physical and chemical barriers, as well as inducible defense responses that become activated upon pathogen infection or insect herbivory. A concerted action of these defensive activities helps the plant to minimize damage caused by the attacker. Many studies have indicated that jasmonic acid (JA) and its derivatives are the most important regulators of induced resistance against herbivore attack. A classic example is the observation that following attack by larvae of the tobacco hornworm, *Manduca sexta*, tomato leaves accumulate JA, resulting in the activation of genes encoding proteinase inhibitors that inhibit digestive serine proteinases of herbivorous insects and reduce further insect feeding (Howe, 2005; Ryan, 2000). Genetic evidence demonstrates that JAs also play an important role in induced defense against

different types of herbivores in Arabidopsis (Ellis *et al.*, 2002; McConn *et al.*, 1997; Reymond *et al.*, 2004; Stintzi *et al.*, 2001; Stotz *et al.*, 2002; Van Poecke and Dicke, 2004). Analysis of the transcriptome of Arabidopsis upon infestation by larvae of the cabbage white butterfly, *Pieris rapae*, revealed that the majority of the induced changes in gene expression is regulated by JA (De Vos *et al.*, 2005; Reymond *et al.*, 2000; 2004). Among the JA-responsive genes that are activated several encode transcription factors, including several members of the MYB-transcription factor family. However, their role in induced resistance against insects is unknown.

MYB genes encode transcription factor proteins that share the conserved MYB DNA-binding domain (Jin and Martin, 1999), and were first identified as oncogenes derived from retroviruses in animal cells (Klempnauer *et al.*, 1982). MYB proteins are categorized into subfamilies depending on the number of conserved MYB domain repeats. MYB proteins from animals generally contain three MYB repeats, which are referred to as R1, R2 and R3. Most of the MYB-like genes in plants have only the R2 and R3 repeats. An inventory of the Arabidopsis genome revealed that this plant species contains approximately 125 R2R3-MYB genes (Stracke *et al.*, 2001). R2R3-MYB proteins in plants have been implicated in a range of activities, such as plant secondary metabolism, regulation of cell death, stress tolerance (reviewed in Stracke *et al.*, 2001), and pathogen resistance, but the functions of most of them have not been determined. The family of R2R3-MYB-like transcription factors has repeatedly been implicated in JA-dependent defense responses. For instance, the *OsLTR1* gene from rice regulates JA-dependent defense responses, whereas AtMYB15 and AtMYB51 are associated with the wound response (Cheong *et al.*, 2002). In addition, Mengiste *et al.* (2003) demonstrated a role for the R2R3-MYB transcription factor protein BOS1 (AtMYB108) in resistance against the necrotrophic pathogens *Botrytis cinerea* and *Alternaria brassicicola*, both of which are sensitive to JA-dependent defense responses (Thomma *et al.*, 1998; Ton *et al.*, 2002). Pathogen-induced expression of AtMYB108 was impaired in the JA-response mutant *coi1*, indicating that AtMYB108 is regulated by JA. Interestingly, AtMYB108 knockout mutants were not only impaired in resistance against necrotrophic pathogens, but also displayed impaired tolerance against water deficit and salt stress (Mengiste *et al.*, 2003). These observations suggest that AtMYB108 is a central player in multiple stress responses in Arabidopsis. Recently, AtMYB72 was demonstrated to be essential for the onset of rhizobacteria-induced systemic resistance (ISR), a JA-dependent induced defense response that is effective against a broad spectrum of plant pathogens (Pieterse *et al.*, 2002; Van Loon *et al.*, 1998). Colonization of the roots by ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria led to the activation of the AtMYB72 gene in the roots (Verhagen *et al.*, 2004).

AtMYB72 knockout mutants were no longer able to express ISR in the leaves, indicating that AtMYB72 is an important regulator of ISR (Verhagen, 2004).

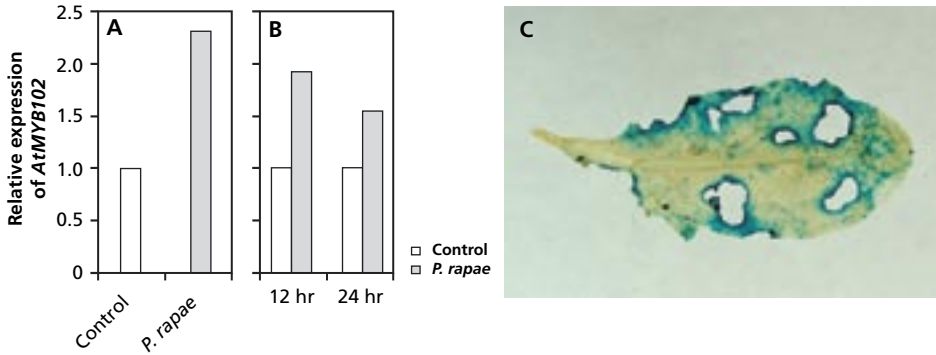
Another R2R3-MYB transcription factor family member (AtMYB102) was identified from an Arabidopsis transcription factor collection (Quaedvlieg *et al.*, 1996). The gene is up-regulated in Arabidopsis upon treatment with ABA, JA, or a combined treatment of osmotic stress and wounding (Denekamp and Smeekens, 2003). Plant responses that are triggered by feeding insects partly overlap with those activated upon dehydration stress and wounding (Reymond *et al.*, 2000). This prompted us to study in how far *AtMYB102* is involved in the response of Arabidopsis to feeding larvae of the specialist herbivore *P. rapae*. Here, we provide evidence that AtMYB102 plays a role in resistance against these tissue-chewing caterpillars and may regulate multiple genes that are involved in cell wall modification.

## Results and discussion

### ***AtMYB102* expression upon herbivore attack**

Herbivore-infested plants undergo substantial transcriptional reorganization in which the plant hormone jasmonic acid (JA) plays an important regulatory role (De Vos *et al.*, 2005; Reymond *et al.*, 2000; 2004). The transcription factor gene *AtMYB102* is induced by dehydration and wounding (Denekamp and Smeekens, 2003). Because herbivore-damaged plants also suffer from water loss, we investigated the role of AtMYB102 in defense against caterpillar feeding. Wild-type Arabidopsis Col-0 plants were infested with larvae of *P. rapae* and the expression of *AtMYB102* was analyzed 24 hr later. Q-RT-PCR analysis of *AtMYB102* mRNA levels showed a 2.3-fold induction of *AtMYB102* in *P. rapae*-damaged tissue compared to untreated Col-0 plants (Fig. 1A), indicating that insect feeding induced the expression of *AtMYB102*. This result was confirmed by data from a previously published whole-genome GeneChip array experiment (De Vos *et al.*, 2005), in which *AtMYB102* mRNA levels were increased at both 12 hr and 24 hr after infestation by *P. rapae* (Fig. 1B).

To further study the herbivore-induced expression of *AtMYB102*, we made use of a transgenic *AtMYB102:GUS* reporter line, containing a translational fusion of the *uidA* reporter gene with the promoter of the *AtMYB102* gene (Denekamp, 2001). Figure 1C shows that m-glucuronidase (GUS) activity was strongly induced around the feeding sites of *P. rapae*. All together, these results indicate that wounding caused by feeding of *P. rapae* triggers the expression of *AtMYB102*, predominantly in the cells surrounding the feeding sites.



**Figure 1.** *Pieris rapae*-induced expression of AtMYB102

(A) Q-RT-PCR analysis of *AtMYB102* mRNA levels in Col-0 plants 24 hr after feeding by first-instar larvae of *P. rapae*. Uninfested control is set at 1.

(B) Relative level of *AtMYB102* mRNA in Col-0 plants 12 and 24 hr after *P. rapae* feeding. Values are derived from an Affymetrix ATH1 GeneChip experiment (De Vos *et al.*, 2005). Uninfested control is set at 1.

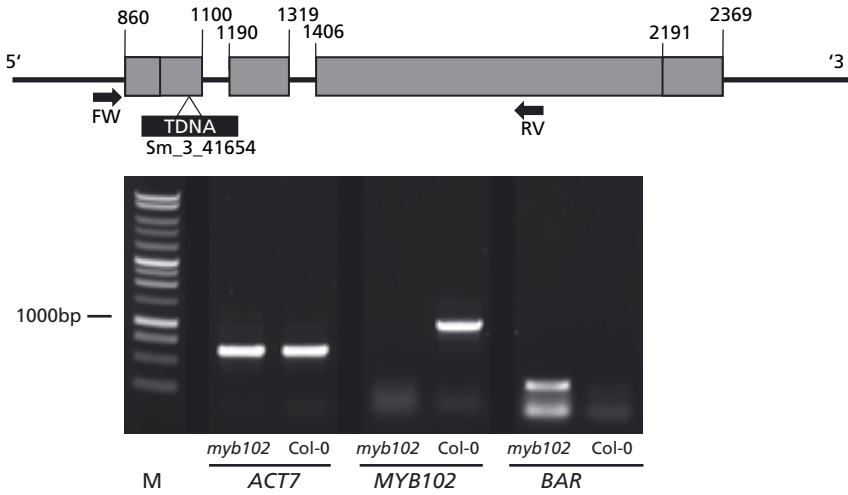
(C) Histochemical staining of  $\beta$ -glucuronidase (GUS) activity in leaves of transgenic Arabidopsis line *MYB102:GUS* 24 hr after feeding by *P. rapae*.

### Role of AtMYB102 in resistance against *P. rapae*

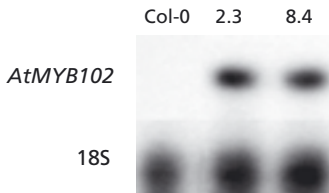
To investigate the role of AtMYB102 in resistance against *P. rapae*, an *AtMYB102* T-DNA insertion line (designated *myb102*), and a 35S:*MYB102* over-expressing line (line 2.3; Denekamp, 2001) were used. Knockout mutant *myb102* contains a T-DNA insertion with a selectable marker for resistance against the herbicide glufosinate (*BAR*) in the first exon of the *AtMYB102* gene (Fig 2A). To confirm disruption of *AtMYB102* in *myb102*, gene-specific primers for *AtMYB102*, the non-target gene *AtACT7*, and the *BAR* gene were used to amplify the respective target sequences in Col-0 and *myb102*. The *AtACT7* gene was detected in the *myb102* mutant as well as in wild-type Col-0 plants (Fig. 2A). The *AtMYB102* primers did not amplify a PCR product in the *myb102* mutant, presumably because of the presence of the large T-DNA insert. The PCR reaction with primers for the *BAR* gene confirmed the presence of a T-DNA insertion in *myb102*. Over-expression of *AtMYB102* in 35S:*MYB102* line 2.3 was confirmed by northern blot analysis of RNA that was isolated from uninduced wild-type and transgenic plants (Fig. 2B).

To study herbivore performance in the knockout mutant and the over-expressor in comparison to wild-type Col-0, 5-week-old plants were infested with 1 freshly hatched *P. rapae* larva. Subsequently, larval performance was monitored over a period up to 10 days by determining larval weight gain. In addition, we determined the percentage of larvae that pupated within 14 days of infestation. Figure 3A shows that on days 7 and 10, the weight of the larvae that fed on *myb102* was significantly higher (approx. 1.5-fold) than that of the larvae feeding on wild-type Col-0 plants. This increased caterpillar weight was

**A AtMYB102 (At4g21440)**



**B**

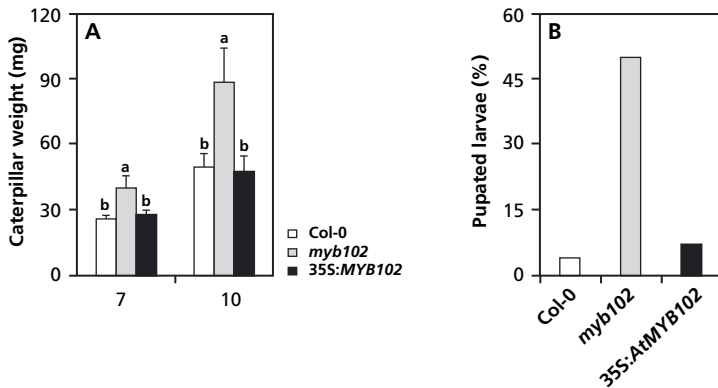


**Figure 2.** Molecular analysis of knockout mutant *myb102* and *AtMYB102* over-expressor *35S:MYB102* lines.

**(A)** Structure of the *AtMYB102* gene and position of the T-DNA insertion in the *myb102* mutant allele. Exons are indicated as gray boxes. The nucleotide numbers above indicate the start and the end of the exons. A T-DNA insertion in *myb102* is located in the first exon of the *AtMYB102* open reading frame. The primers used for the verification of the position of the T-DNA insertion are indicated by arrows (FW and RV). The expected size of the PCR products for Col-0 is 1002 bp. FW, *AtMYB102* forward primer; RV, *AtMYB102* reverse primer. To verify the T-DNA insertion, PCR amplification of genomic DNA of Col-0 and *myb102* plants was performed using the *AtMYB102*-specific FW and RV. Specific primers for *AtACT7* were designed as internal loading control. M = 1000bp DNA ladder.

**(B)** Northern blot analysis of *AtMYB102* mRNA levels in *35S:MYB102* lines 2.3 and 8.4. The blot was hybridized with a gene-specific probe for *AtMYB102*. The probe for 18S rRNA was used to check for equal loading.

associated with a greater percentage of larvae that had entered pupation by day 14. About 50% of the larvae feeding from *myb102* plants had developed into pupae on day 14, while only 5% of the larvae feeding from wild-type Col-0 plants had pupated on that time point (Fig. 3B). Surprisingly, over-expression of *AtMYB102* did not result in a reduction of larval performance. Caterpillar growth on *35S:MYB102* plants did not differ significantly from that on Col-0 plants (Fig. 3A). Also the percentage of larvae feeding from



**Figure 3.** Effect of herbivore-induced resistance on *P. rapae* performance

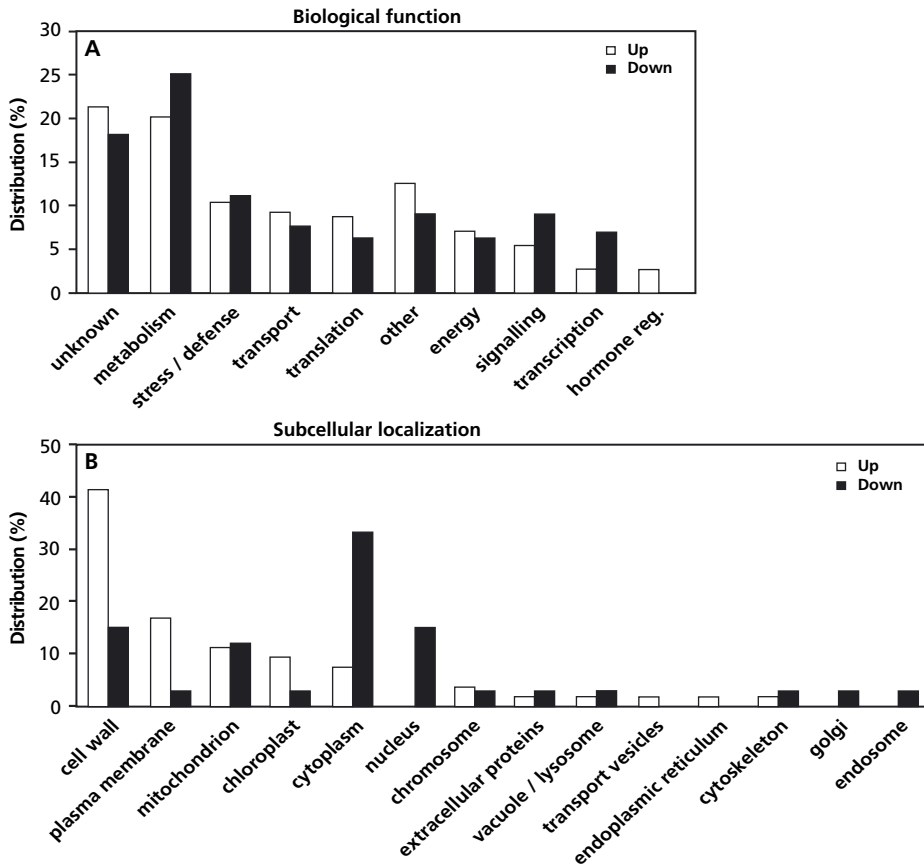
**(A)** Growth of *P. rapae* larvae on wild-type Col-0, mutant *myb102*, and *AtMYB102* over-expressing *35S:MYB102* plants. Larval fresh weight (FW) was measured after 7 and 10 days of feeding. The values presented are means ( $\pm$ SE) of 20 larvae on each plant genotype. Different letters indicate statistically significant differences between treatments (Fisher's LSD test;  $\alpha=0.05$ ).

**(B)** Percentage of *P. rapae* larvae ( $n=20$ ) that had developed into pupae by 14 days after infestation (DAI).

*35S:MYB102* plants that had pupated by day 14 did not differ significantly from those feeding on wild-type Col-0 plants (Fig. 3B). These data indicate that *AtMYB102* contributes to basal resistance against *P. rapae* feeding. However, over-expression of *AtMYB102* does not increase resistance above the basal level.

### Expression profiling of *35S:MYB102* plants

To study downstream effects of up-regulation of *AtMYB102* by *P. rapae* we performed a microarray experiment to identify the genes that are regulated by the transcription factor *AtMYB102*. To this end, wild-type Col-0 plants and *35S:MYB102* line 8.4 (Denekamp, 2001) were compared in a dedicated cDNA micro-array consisting of approximately 6,000 Arabidopsis cDNA fragments. Over-expression of *AtMYB102* significantly increased the expression ( $>2$ -fold) of 151 genes, while 117 genes showed an at least 2-fold reduction (supplementary data Table S1). We categorized the differentially expressed genes according to biological function (Fig. 4A) and predicted subcellular localization (Fig. 4B) using internet tools from the MIPS *Arabidopsis thaliana* Genome Database (MatDB; <http://mips.gsf.de/proj/thal/db/index.html>) and the Gene Ontology tool at TAIR (<http://arabidopsis.org/tools/bulk/go/index.jsp>). Classification according to biological functions indicates that a substantial percentage of the differentially expressed genes in the *35S:MYB102* over-expressing line encode proteins involved in metabolism. However, this is not surprising because of all annotated genes in the Arabidopsis genome, metabolism is the largest category of genes with known biological function. Moreover, 10% of the genes up- and



**Figure 4.** Biological function and predicted cellular localization of the proteins encoded by the differentially expressed genes in 35S:MYB102 plants.

down-regulated by over-expression of *AtMYB102* have been shown to be involved in stress and defense reactions. Classification according to predicted subcellular localization of the proteins revealed that a large proportion of the up-regulated genes encode proteins that are thought to function in the cell wall or at the plasma membrane. Among the up-regulated genes are several that code for cell wall-modifying proteins, such as EXPANSIN4, 8, 10 and 11, and pectolytic enzymes (Table 1).

## Conclusions

In this study we showed that damage caused by feeding larvae of *P. rapae* induced the expression of *AtMYB102* around the feeding sites. On knockout *myb102* plants, *P. rapae* caterpillars developed significantly faster than on Col-0 plants, indicating that in wild-type plants *AtMYB102* plays a role in defense

**Table 1.** Fold-change ratio of up-regulated genes that are associated with modification of the cell wall upon over-expression of *AtMYB102* in Arabidopsis.

Function <sup>1</sup>	Annotation	AGI No.	Fold-change <sup>2</sup>
<b>Biosynthesis</b>			
- Cuticle biosynthesis	$\alpha$ -keto acyl reductase	At1g67730	2.04
- Cell wall organization	Copia-like retrotransposon family Protodermal factor 1	At2g06950	3.34
		At2g42840	2.36
<b>Degradation</b>			
- Cell wall loosening	Expansin (EXP11)	At1g20190	5.48
	Expansin (EXP10)	At1g26770	4.10
	Expansin (EXP8)	At2g40610	3.21
	Expansin (EXP4)	At2g39700	2.69
- Pectin degradation	Pectinesterase	At1g14890	2.52
	Pectinesterase	At1g11580	2.39
	Pectate lyase	At1g04680	2.05
- Xyloglucan cleavage	Endo-xyloglucan transferase	At2g06850	3.58
- Cellulase	Endo-1,4- $\alpha$ -glucanase	At1g70710	2.86
- $\alpha$ -glucosidase	$\alpha$ -glucosidase activity	At3g09260	3.07

<sup>1</sup> Described functions are based on the Gene Ontology tool at the TAIR internet facilities

<sup>2</sup> Fold-change ratios (35S:*MYB102*/Col-0) are based on gene expression profiles of 5-week-old leaf tissue from 35S:*MYB102* line 8.4 and wild-type Col-0 plants.

against this herbivore. Over-expression of the *AtMYB102* gene did not further enhance the level of resistance against *P. rapae* feeding, suggesting that the level of *AtMYB102* that is induced upon herbivore feeding is already fully effective. Transcript profiling of wild-type Col-0 and *AtMYB102* over-expressing plants revealed that a large proportion of genes that were significantly up-regulated in the *AtMYB102* over-expressor are predicted to exert their function in the cell wall or the plasma membrane. Moreover, several genes involved in cell wall remodeling were up-regulated in the *AtMYB102* over-expressor. Our findings that *AtMYB102* plays a role in resistance against *P. rapae*, and regulates genes that are associated with cell wall modification, raises the question in how far a causal relationship exist between these two processes. The speed of tissue consumption by *P. rapae* suggests that the cell wall modifications that are induced upon activation of *AtMYB102* are unlikely to contribute to inhibition of growth of the caterpillars. Hence, the *AtMYB102*-mediated cell wall modifications may reflect repair mechanisms that are initiated upon wounding and dehydration. However, knockout mutant *myb102* clearly allows a faster development of *P. rapae* larvae, indicating that *AtMYB102*-regulated genes contribute to resistance against this herbivore. Clearly, more research

is required to understand the role of AtMYB102 in resistance against insect feeding.

## Materials and methods

### Cultivation of plants

Seeds of *Arabidopsis thaliana* accession Col-0, *MYB102:GUS* (Denekamp and Smeekens, 2003), knockout mutant *myb102* (T-DNA insertion line Smm3m41654 obtained from the EXOTIC collection of the Nottingham Arabidopsis Stock Centre; Tissier *et al.*, 1999) and *AtMYB102* over-expressing 35S:*MYB102* plants (line 2.3; Denekamp, 2001) were sown in quartz sand. All genotypes were in the Col-0 background. Two-week-old seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min. Plants were cultivated in a growth chamber with a 8-hr day (200  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  at 24°C) and 16-hr night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing 10  $\mu\text{M}$  Sequestreen (CIBA-Geigy, Basel, Switzerland), once a week. For the microarray analysis, Col-0 and 35S:*MYB102* plants were grown until they had reached the same developmental stage (fully mature rosettes that had not started bolting; 5 weeks for Col-0 and 8 weeks for 35S:*MYB102*) in potting soil in a growth chamber with a 8-hr day (24°C) and a 16-hr night (20°C) cycle at 70% humidity. Instead of line 2.3 (which was used for the insect bioassays), 35S:*MYB102* line 8.4 was used for the microarray analysis. Both *AtMYB102* over-expressing lines showed similar levels of *AtMYB102* mRNA (Denekamp, 2001). Line 2.3 was used in the insect bioassays because at the time of the insect experiments, no viable seeds could be recovered from line 8.4.

### Insect bioassay

Tissue-chewing larvae of the small cabbage white butterfly, *Pieris rapae*, were reared on Brussels sprout plants (*Brassica oleracea gemmifera* cv. *Cyrus*) in a growth chamber with a 16-hr day and 8-hr night cycle (21°C; 50–70% relative humidity) as described previously (Van Poecke *et al.*, 2001). Infestation of Arabidopsis plants was carried out by transferring first-instar larvae to 5-week-old plants using a fine paintbrush. To study *P. rapae* performance, a single freshly hatched first-instar larva was transferred to each of 20 Col-0, *myb102*, or 35S:*MYB102* plants. At 7 and 10 days, the fresh weight of each larva was determined. After 10 days, the first larvae started to pupate. Therefore, fresh weight was determined only up to 10 days of feeding. To examine effects on

caterpillar development, the percentage of caterpillars that had pupated within 14 days after hatching was determined.

#### Confirming T-DNA insertion in *AtMYB102*

Seedlings from Col-0 and T-DNA insertion line Sm\_3\_41654 were grown for two weeks on Murashige and Skoog (MS) medium supplemented or not with 20 mg.L<sup>-1</sup> BASTA and subsequently harvested for isolation of genomic DNA. Disruption of the *AtMYB102* gene was checked by PCR using gene-specific primers for *AtMYB102* (At4g21440; FOR 5'-TTC CCC TTA CGG ACC CTA CGA-3'; REV 5'-TGG TGG CAT GGA AGA TTG GAG T-3') located on opposite sites of the predicted T-DNA insertion. Gene-specific primers for *AtACT7* (At5g09810; FOR; 5'-GAC ATG GAA AAG ATA TGG CAT CAC AC-3'; REV; 5'-AGA TCC TTC CTG ATA TCG ACA TCA C-3'), and *BAR* (FOR; 5'-ACT TCA GCA GGT GGG TGT AGA G-3'; REV; 5'-ATC GTC AAC CAC TAC ATC GAG AC-3') were used as controls. The following PCR program was used for all PCR reactions: 95 °C for 3 min; 40 cycles of 92 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min.

#### Quantitative real-time PCR

Q-RT-PCR analysis was performed basically as described previously (Czechowski *et al.*, 2004). Two µg of RNA was digested with Turbo DNA-free™ (Ambion, Huntingdon, United Kingdom) according to the manufacturer's instructions. To check for genomic DNA contamination, a PCR with primers designed on intron sequences of *AtACT7* (At5g09810; *ACT7*-FOR: 5'-GAC ATG GAA AAG ATA TGG CAT CAC AC-3'; *ACT7*-REV: 5'-AGA TCC TTC CTG ATA TCG ACA TCA C-3') was carried out. Subsequently, DNA-free total RNA was converted into cDNA using oligo-dT<sub>20</sub> primers (Invitrogen, Breda, the Netherlands), 10 mM dNTPs, and SuperScript™ III Reverse Transcriptase (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Efficiency of cDNA synthesis was assessed by Q-RT-PCR, using primers of the constitutively expressed gene *AtUBI10* (At4g05320; *UBI10*-FOR: 5' AAA GAG ATA ACA GGA ACG GAA ACA TAG T-3'; *UBI10*-REV: 5'-GGC CTT GTA TAA TCC CTG ATG AAT AAG-3'). Gene-specific primers were designed for *AtMYB102* (At4g21440; *AtMYB102*-FOR: 5'-GTT GCC AGA AGA ACG GAC TC-3'; *AtMYB102*-REV: 5'-GGG AGG GTT CTC CAG TTA CC-3'). Q-RT-PCR analysis was done in optical 96-well plates with an MyIQ™ SingleColor Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands), using SYBR® Green to monitor dsDNA synthesis. Each reaction contained 1 µL of cDNA, 0.5 µL of each of the two gene-specific primers (10 pmol.µL<sup>-1</sup>), and 10 mL of 2x IQ SYBR® Green Supermix reagent (Bio-Rad, Veenendaal, the Netherlands) in

a final volume of 20  $\mu$ l. The following PCR program was used for all PCR reactions: 95 °C for 3 min; 40 cycles of 95 °C for 30 sec, 59.5 °C for 30 sec, and 72 °C for 30 sec.  $C_T$  (threshold cycle) values were calculated using Optical System Software, version 1.0 for MyIQ™ (Bio-Rad, Veenendaal, the Netherlands). Subsequently,  $C_T$  values were normalized for differences in dsDNA synthesis using the *AtUBI10*  $C_T$  values. Normalized transcript levels of *AtMYB102* were compared to untreated controls and the fold change in expression level was calculated after 24 hr of feeding by *P. rapae*.

### **GUS assay**

Larvae of *P. rapae* were transferred to 5-week-old *MYB102:GUS* plants. After 24 hr of caterpillar feeding, leaf tissue was harvested and GUS activity assessed by transferring the leaves to GUS staining solution (1 mM X-Gluc, 100 mM NaPi buffer, pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM potassium ferrocyanide and 1 mM potassium ferricyanide). After overnight incubation at 37 °C, the leaves were destained by repeated washes in 70% ethanol and evaluated for staining intensity.

### **Sample preparation and microarray analysis**

For isolation of RNA from leaf tissue, shoots of untreated wild-type Col-0 and 35S:*MYB102* line 8.4 plants were harvested. Total RNA (5  $\mu$ g) of each sample was reverse transcribed and amplified according to a modified protocol for *in vitro* transcription (<http://www.microarrays.be/service.htm>), labeled with fluorescent Cy5 or Cy3 (Amersham Biosciences, Roosendaal, the Netherlands), and subsequently hybridized to a dedicated Arabidopsis 6K microarray consisting of 6,008 cDNA fragments. Fragments and controls were obtained from the Incyte Unigene collection (Arabidopsis Gem I; Incyte, Palo Alto, CA) and the Universal Score Card spike set (Amersham BioSciences, Little Chalfont, UK), respectively. Clones were spotted in duplicate, distant from each other (for details see <http://www.microarrays.be/service.htm>).

Hybridization and washing were performed in an automated hybridization station (Amersham Biosciences, Roosendaal, the Netherlands). The arrays were scanned at 532 and 635 nm by a Generation III scanner (Amersham BioSciences, Roosendaal, the Netherlands) and images were analyzed with an ArrayVision (Imaging Research Inc, Ontario, Canada). Genes showing at a least 2-fold change in expression (increase or decrease in line 8.4 compared to wild-type plants) were annotated using the MIPS *Arabidopsis thaliana* Genome Database (MatDB; <http://mips.gsf.de/proj/thal/db/index.html>). Biological function and predicted subcellular localization of the proteins was assessed using the same Internet facilities and the Gene Ontology tool at TAIR.

## Acknowledgements

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**Supplementary Table 1:** Selected genes showing an at least 2-fold difference in expression between Col-0 and *AtMYB102* over-expressing (35S:*MYB102*) plants. This supplementary table can be found at [http://www.bio.uu.nl/~fytopath/GeneChip\\_data.htm](http://www.bio.uu.nl/~fytopath/GeneChip_data.htm).



## General discussion

### Plants under attack

Plants are sessile organisms that are under constant threat of possible invaders. Despite the fact that these potentially harmful organisms are highly abundant, plants are resistant to most micro-organisms and insects encountered. This type of resistance is known as non-host resistance and consists of constitutive physical and chemical barriers that are effective against a broad range of possible invaders (Mysore and Ryu, 2004; Thordal-Christensen, 2003).

In case pathogenic micro-organisms or insects are able to invade the plant, it will mount inducible defense responses. As a first line of defense, plants use basal defenses, which are regulated by several plant hormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (reviewed by Glazebrook, 2005). In addition, depending on the genetic constitution of the host plant other defenses can be mobilized. These induced defense responses are dependent on a specific recognition of the invader and a rapid induction of a range of effective defense-related mechanisms. *R*-gene-mediated resistance is the best-characterized induced defense response. Upon recognition of a pathogen-derived molecule, that is associated with the activity of an avirulence (effector) gene, by a corresponding resistance gene in the host, a quick reaction is triggered that limits pathogen growth. This response, which usually takes the form of an hypersensitive reaction, limits infection to a restricted area of a few cells undergoing apoptosis (Dangl and Jones, 2001). Other induced responses result in enhanced resistance throughout the whole plant. These are triggered through plant-derived signaling molecules and require a complex signal-transduction pathway. SA-, JA-, and ET-dependent responses have been associated with these types of induced resistance as well. Although exceptions have been described (Thaler *et al.*, 2004), a distinction between SA-dependent resistance against biotrophic pathogens and JA-dependent resistance to necrotrophic pathogens has been proposed (Glazebrook, 2005; Thomma *et al.*, 2001; Ton *et al.*, 2002). This subdivision of defense responses is based on the

increased susceptibility of SA- or JA-impaired *Arabidopsis* plants to several pathogens and the spectrum of resistance observed upon exogenous application of either SA, JA, or their functional analogues.

Two well-known induced defense responses that extend systemically to non-infected plant parts and confer a partly resistant phenotype also depend on a functional SA- or JA-signaling pathway. For instance, earlier results demonstrated that systemic acquired resistance (SAR) requires an accumulation of SA for enhanced resistance against biotrophic pathogens (Durrant and Dong, 2004). Conversely, rhizobacteria-mediated induced systemic resistance (ISR) is effective against pathogens that are restricted through JA-dependent defense responses (Ton *et al.*, 2002). These results suggest that induced defenses against pathogens are reinforcements of extant SA- or JA-dependent basal defense responses (Ton *et al.*, 2002).

Wound responses are triggered upon mechanical damage or feeding by herbivorous insects. These induced defenses have been shown to depend predominantly on increased JA levels. Subsequent JA-responsive gene expression leads to the accumulation of toxic, anti-nutritional, or repellent compounds. A classic example is the JA-inducible accumulation of proteinase inhibitors in tomato upon feeding by herbivores (Howe, 2005; Ryan, 2000). These interfere with the digestive activity of the insect (Pearce *et al.*, 1991), reduce feeding, and prolong the time that the insect is vulnerable to parasitoids and carnivorous predators (Kessler and Baldwin, 2004). Like in tomato, induced defense against herbivores in *Arabidopsis* is JA-dependent. JA-impaired mutants have been shown to be more susceptible to insect feeding by many *Lepidoptera* species (McConn *et al.*, 1997; Reymond *et al.*, 2004; Stotz *et al.*, 2002).

Resistance against microbial pathogens and herbivorous insects is a costly investment. Constitutive defenses are immediately effective upon attack, but there is a trade-off penalty with regard to growth and fitness of the plant (Baldwin, 1998; Heidel and Baldwin, 2004; Heil *et al.*, 2000). Induced defenses require less investment and are, therefore, more cost-efficient, as they are triggered only upon attack. Some induced defenses consist of a primed state, in which defense responses are activated faster and stronger upon attack (Conrath *et al.*, 2002). For example, Verhagen *et al.* (2004) demonstrated that rhizobacteria-mediated ISR is associated with priming for JA-responsive gene expression, which is likely to proceed through upregulation of transcription factors, which make ISR effective against a broad spectrum of pathogens.

Indeed, plants are often resistant to pathogen and insect attack and it seems that susceptibility to infection by pathogens or infestation by herbivorous insects is a rarity. In case the plant-attacker combination leads to infection, plants have the ability to induce multiple defense mechanisms, which in many cases restrict further pathogen growth or insect development. Many plant pathogens

and herbivorous insects are able to infect their host plants because they are specialized and have found ways to circumvent the defense mechanisms of their hosts.

## **Unraveling the complexity of the plant's induced defense signaling network**

An important question in plant defense signaling research is: how are plants capable of integrating signals produced upon attack by pathogenic micro-organisms or feeding by insects into defenses that are specifically directed against the invader encountered? While the importance of SA, JA, and ET in plant defense is clear, evidence is accumulating that their signaling pathways cross-communicate to provide the plant with a powerful regulatory potential, which can help the plant to “decide” which defensive strategy to follow (Dicke and Van Poecke, 2002; Felton and Korth, 2000; Feys and Parker, 2000; Kunkel and Brooks, 2002; Pieterse and Van Loon, 1999; Pieterse *et al.*, 2001; Reymond and Farmer, 1998; Rojo *et al.*, 2003). The defense response that is subsequently expressed is directed against the invader encountered, but what are the consequences for resistance against other types of pathogens or insects? Because SA- and JA-dependent defenses are often mutually exclusive (Bostock, 2005; Pieterse *et al.*, 2001; Spoel *et al.*, 2003), it is tempting to speculate that the SA-dependent induced defense response that is triggered upon infection by necrotizing pathogens, and is predominantly effective against biotrophic pathogens, would counteract JA-dependent defenses that are effective predominantly against necrotrophic pathogens and insect feeding. Conversely, JA-dependent defenses, such as triggered upon insect feeding, are likely to be effective against herbivores and necrotrophic pathogens, but would impede resistance against biotrophic pathogens. Detailed knowledge of the nature of the defense response that is triggered upon pathogen or insect attack, and the spectrum of effectiveness of the associated induced resistance, would greatly contribute to our understanding of how the plant's innate immune response is functioning.

## **Attacker-specific transcriptome changes in *Arabidopsis***

Induced plant defenses upon attack have long been characterized by analysis of marker gene expression and their encoded proteins, such as pathogenesis-related (PR) proteins during SAR. With the development of large-scale gene-expression analysis, such as cDNA-AFLP (Bachem *et al.*, 1996) and DNA micro-array technology (Schena *et al.*, 1995), it became possible to study

simultaneously the expression of thousands of genes. The latter technique has now been optimized and been adopted by many researchers. From the moment that the Arabidopsis genome sequence was established (Kaul *et al.*, 2000), gene expression studies of Arabidopsis under attack by pathogens and herbivorous insects have been published (Glazebrook *et al.*, 2003; Moran *et al.*, 2002; Reymond *et al.*, 2000; 2004; Tao *et al.*, 2004; Van Wees *et al.*, 2003; Verhagen *et al.*, 2004). Many of these studies are conducted using Affymetrix full-genome arrays (approx. 23,750 genes). For instance, Tao *et al.* (2003) demonstrated that the response to infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* in the absence of *R*-gene-mediated recognition (virulent *P. syringae*) leads to a similar transcription profile as infection with an avirulent strain. Although similar, the responses to virulent *P. syringae* occur later, which can explain that these are less effective in limiting the infection (Tao *et al.*, 2003). Analysis of the interaction between Arabidopsis and the fungal pathogen *Alternaria brassicicola* is studied preferentially in the phytoalexin-deficient mutant *pad3-1*, which in contrast to wild-type Col-0 plants is susceptible to this fungus (Thomma *et al.*, 1998). Recently, Van Wees *et al.* (2003) analyzed whole-genome expression profiles of Arabidopsis upon *A. brassicicola* infection. Not surprisingly, they found a great overlap in early gene expression (up to 36 hr) between Col-0 and *pad3-1*, which suggests that the mutation in *pad3-1* does not affect signaling upon infection and is disturbed only in the production of the phytoalexin camalexin.

Because in most studies the experimental set-up, such as growth conditions, time points after inoculation, and the age of the plant material at harvest, varies, we decided to investigate the transcriptional changes upon infection with several pathogenic micro-organisms and herbivorous insects with distinct feeding strategies under comparable conditions. Each of these interactions, *P. syringae*, *A. brassicicola*, *Pieris rapae*, *Frankliniella occidentalis*, and *Myzus persicae*, resulted in an attacker-specific damage pattern (Chapter 2; Fig. 1). Moreover, results from this comparative study showed that Arabidopsis reacts to the invasion by various attackers with specific blends of signaling molecules (SA, JA, and ET). These blends vary in composition, timing, and amplitude, and are specific for each Arabidopsis-attacker combination (Chapter 2; Fig. 2). Furthermore, transcriptome analysis of each Arabidopsis-attacker combination revealed that the plants are highly flexible in adapting to these attackers. Each Arabidopsis-attacker combination leads to an attacker-specific gene expression profile (Chapter 2; Table 3). Interestingly, despite the fact that JA was produced in four out of the five interactions studied, this still led to an attacker-specific transcriptome profile of which the overlap between interactions ranged between 6–54% (Chapter 2; Table 4). This was particularly striking in the interactions of Arabidopsis with *A. brassicicola*, *P. rapae*, and *F. occidentalis*. In all three

interactions, JA was the dominant signaling molecule produced upon attack, and up to 69% of all genes with consistent changes in expression were responsive to JA. However, pair-wise comparisons revealed that 46–96% of the consistent JA-responsive changes are expressed in an attacker specific manner. This suggests that, although JA is a dominant primary signal molecule in these Arabidopsis-attacker combinations, additional layers of regulation shape the final outcome of the defense response.

### **Cross-resistance: it is not that obvious**

The flexibility of a plant in responding to pathogen or insect attack, raises questions about the specificity of the induced defense responses that are triggered. For instance, are induced defenses that are triggered upon herbivore feeding specifically directed against herbivores, or do they provide cross-resistance against certain pathogens as well? Cross-resistance between feeding by herbivorous insects and infections by pathogens has been observed in many plant species, including several crop plants (Karban and Baldwin, 1997). Plant growth and physiology are substantially changed upon attack by either microbial pathogens or herbivorous insects. These changes, in turn, can alter the suitability for subsequent attack by subsequent invaders. For instance, upon wounding, plants become more susceptible to opportunistic micro-organisms that are unable to infect healthy plants (Agrios, 2005). On the other hand, water relations and nutrient composition change upon primary attack, which affects the quality of the food source for subsequent attackers. For example, Hatcher *et al.* (1995) reviewed the changes in accumulation of photoassimilates and protein, amino acid, and nutrient content in the three-way interaction between a leaf beetle *Gastrophysa viridula*, the biotrophic rust fungus *Uromyces rumicis*, and their common host plant *Rumex obtusifolius*. Adult beetles prefer feeding on healthy plants. Moreover, oviposition behavior was negatively influenced by rust infection. In contrast, peanut plants infected by white mold (*Sclerotium rolfsii*) were consumed to a larger extent by larvae of the beet armyworm, *Spodoptera exigua* (Cardoza *et al.*, 2002). In tomato, infection by the corn earworm (*Helicoverpa zea*) reduces proliferation of the bacterial pathogen *P. syringae* pv. *tomato*, and vice versa (Stout *et al.*, 1999). Because some of these data are contradictory, it is a challenge to understand the regulatory mechanisms underlying cross-resistance (Rostas *et al.*, 2003).

Cross-resistance between induced defenses against microbial pathogens has been well established (Hammerschmidt and Kuc, 1995). For instance, SAR triggered upon recognition of an avirulent pathogen has been shown to be effective against a wide range of pathogens (Kuc, 1987). Moreover, non-pathogenic rhizobacteria-mediated ISR has been shown to be effective against

a broad range of microbial pathogens, including bacteria, fungi, and oomycetous pathogens (Pieterse *et al.*, 1996; Ton *et al.*, 2002). Similar mechanisms triggered by herbivore feeding have been described showing broad resistance against subsequent insect attack. Recently, Kessler and Baldwin (2004) showed that in tobacco cross-resistance occurs in defense against herbivorous insects. Tobacco plants attacked by the mirid bug, *Tupiocoris notatus*, increased secondary metabolites and proteinase inhibitors to levels that were effective against the tobacco hornworm (*Manduca sexta*). Similarly, feeding of two different herbivores on the roots of *Brassica nigra* induced systemic defense responses against a shoot herbivore, with a different feeding strategy, i.e. *P. rapae*. This specialist caterpillar was affected by increased levels of toxic glucosinolates in the shoots (Van Dam *et al.*, 2005). These results indicate that upon attack by herbivorous insects or pathogenic micro-organisms plants mount resistance responses that are directed primarily against the attacker encountered, but can also influence growth or development of other invaders.

These observations and the involvement of JA in induced defenses against pathogens and insects prompted us to investigate caterpillar-induced resistance in *Arabidopsis* against several microbial pathogens (Chapter 3). We hypothesized that insect-induced resistance is effective against microorganisms that are resisted by similar resistance responses, i.e. JA-inducing larvae of *P. rapae* would increase resistance against pathogens that are restricted through JA-dependent defense responses. *Arabidopsis* is well suited for these types of experiments, because a large number of pathogens and herbivores has been described to attack *Arabidopsis* (Meyerowitz and Sommerville, 1994; Mitchell-Olds, 2001). Moreover, the defense responses upon infection with most of these pathogens have been studied. Mutant analysis and exogenous application of chemicals has provided information on the dependency on signal molecules, such as SA, JA, and ET, for enhanced resistance against these attackers (Glazebrook, 2005; Thomma *et al.*, 1998; Ton *et al.*, 2002). Because the necrotrophic fungus *A. brassicicola* has been shown to be sensitive to JA-dependent defenses, we expected enhanced resistance against this pathogen as a result of feeding by *P. rapae* (Thomma *et al.*, 1998; Ton *et al.*, 2002). Conversely, resistance against turnip crinkle virus (TCV) has been demonstrated to be regulated exclusively by SA (Kachroo *et al.*, 2000; Ton *et al.*, 2002). Therefore, we did not expect any effect on the level of resistance against this biotrophic pathogen. Both expectations appeared to be incorrect (Chapter 3; Fig. 2 and 5). Apparently, other regulating factors influenced the outcome of the induced defense responses. We provided evidence that elicitors in the caterpillar regurgitant actively suppress a branch of the JA signaling pathway that is involved in defense against *A. brassicicola* (exemplified by *PDF1.2* expression (Chapter 3, Fig. 3), thereby explaining the ineffectiveness of herbivore-induced resistance against this pathogen. In

addition, we showed that ET primes the leaf tissue for enhanced expression of SA-inducible defenses that are activated upon infection by TCV (Chapter 3; Fig. 6). Hence, although *P. rapae* feeding is not associated with increased SA levels, herbivore-induced ET production primes the tissue to react faster and more strongly to SA-inducing TCV, leading to enhanced resistance against this pathogen. In addition, we observed that *P. rapae*-induced resistance is effective locally against two bacterial pathogens, *Xanthomonas campestris* and *P. syringae*. Analysis of several mutants impaired in SA-, JA-, and ET-signaling suggested that *P. rapae*-induced local resistance against *P. syringae* does not operate through any of these known pathways (Chapter 3; Fig. 4). Hence, from Chapter 3 it must be concluded that cross-resistance, or the lack of it, is highly unpredictable. Clearly, different regulatory mechanisms, such as pathway cross-talk and priming, are involved in shaping the final outcome of the defense response.

### **Clever attackers: making pathway cross-talk your advantage**

As most microbial pathogens and herbivorous insects cannot successfully attack plants, those that do, have evolved ways to invade the plant tissue. In return, plants are forced to adjust their defenses against adapted pathogenic micro-organisms and herbivorous insects. Specialized attackers have found ways to circumvent recognition by the host plant. Alternatively, they can actively suppress the defense mechanisms used by the host (Kahl *et al.*, 2000). For instance, crucifers deploy a two-component defense system, called ‘the mustard oil bomb’, against herbivorous attackers (Rask *et al.*, 2000). This system, in which glucosinolates and the enzyme myrosinase are stored in separate compartments of the plant cell, is activated when the cells are ruptured upon attack. The myrosinase enzyme cleaves the glucosinolates, releasing toxic isothiocyanates and other repellent volatiles that are effective against many generalist herbivores (Wittstock *et al.*, 2003). There has been some debate in the literature whether specialists, such as *P. rapae* larvae, are susceptible to these glucosinolate break-down products. Although Agrawal and Kurashige (2003) showed that glucosinolates reduced larval survival and development, its butterfly has a strong preference for oviposition on members of the *Brassicaceae* (Karban and Baldwin, 1997). Recently, Wittstock *et al.* (2004) demonstrated that a larval gut protein from *P. rapae* prevents formation of isothiocyanates by redirecting glucosinolate hydrolysis toward nitrile formation. This type of metabolic diversion of chemical host defenses is specific for *P. rapae* caterpillars and can explain their host specificity for cruciferous plants. Other crucifer specialists, such as the cabbage aphid *Brevicoryne brassicae*, are not

only resistant to glucosinolates, but have co-opted this plant defense system to make themselves more resistant to predators (Bridges *et al.*, 2002; Francis *et al.*, 2001; 2002).

Various pathogens can modulate plant signal transduction for their own benefit by taking advantage of the cross-talk between defense signaling pathways. Kloeck *et al.* (2001) showed that the *P. syringae*-derived JA-mimicking phytotoxin, coronatine (COR), acts to promote disease by suppressing SA-dependent defenses. Using both wild-type and coronatine-insensitive *jai1* tomato plants and wild-type and COR-non-producing *P. syringae* pv. *tomato* bacteria, Zhao *et al.* (2003) demonstrated that the causal agent of bacterial speck disease activates the JA signaling pathway to actively suppress the SA-dependent defenses deployed by the host plant. However, application of JA has also been shown to trigger resistance against *P. syringae* in *Arabidopsis* (Pieterse *et al.*, 1998). In addition, JA-impaired mutants other than *coi1-1*, are more susceptible to *P. syringae* infection (Ellis *et al.*, 2002; Pieterse *et al.*, 1998; Ton *et al.*, 2002). Indeed, JA-dependent rhizobacteria-mediated ISR is only effective in plants with an intact JA response (Pieterse *et al.*, 1998; Ton *et al.*, 2002). Thus, JA-mimicking COR can suppress SA-dependent defenses during infection, whereas application of JA prior to infection enhances resistance to *P. syringae* pv. *tomato*.

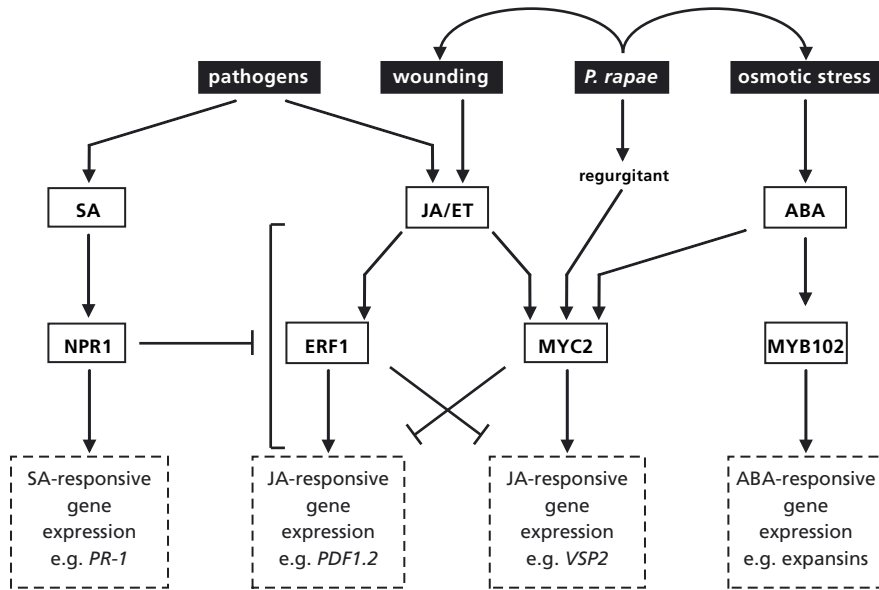
In Chapter 4, we reported that the specialist caterpillar *P. rapae* is able to actively suppress host gene expression. Wound-inducible expression of *PDF1.2* was not triggered by *P. rapae*, as observed also by Reymond *et al.* (2004). Moreover, we demonstrated that a factor present in *P. rapae* regurgitant is involved in the suppression of host defense-related genes (Chapter 4; Fig. 3A and 4A). The mRNA levels of other JA-responsive marker genes, such as *VEGETATIVE STORAGE PROTEIN2 (VSP2)*, *12-OXOPHYTODIENOATE REDUCTASE3 (OPR3)*, and *LIPOXYGENASE2 (LOX2)* were not reduced by *P. rapae* feeding. Comparison of existing Affymetrix ATH1 microarray data sets identified genes that are suppressed by *P. rapae*. These data point to suppression of a specific subset of JA-inducible genes. Wound-induced gene expression branches after the induction of two JA-responsive transcription factors, AtMYC2 and ERF1 (Lorenzo *et al.*, 2004). ERF1 has been shown to regulate the expression of many JA/ET-dependent defense-related genes (Lorenzo *et al.*, 2003), which are down regulated by AtMYC2 (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004). Conversely, genes induced by AtMYC2 are suppressed by ERF1 (Lorenzo *et al.*, 2004). Feeding by *P. rapae* induced AtMYC2 expression and, thereby, suppressed the activation of *PDF1.2* and other defense-related genes (Chapter 4). Moreover, AtMYC2-impaired mutant plants, i.e. *jin1-2*, did not show *P. rapae*-induced suppression of wound-inducible genes, resulting in high *PDF1.2* transcript levels upon herbivore feeding (Chapter 4; Fig. 3A). These results illustrate that

specialist herbivores, such as *P. rapae*, are able to interfere with the host's defense mechanism, and that factor(s) in caterpillar regurgitant are important in this process. Apparently, co-evolution between the host plant and its pests or disease agents allows attackers to manipulate plants for their own benefit by suppressing host defenses through cross-talk interference (Kahl *et al.*, 2000; Zhao *et al.*, 2003; Chapter 4). However, the question whether *P. rapae*-induced down-regulation of defense-related genes, such as *PDF1.2*, is for the benefit of the plant or the attacker, remains unanswered. By prioritizing the *AtMYC2*-activated branch of the JA response the plant may lose resistance against pathogens but gain an enhanced wound response. Alternatively, the branch of the JA response that is suppressed by *AtMYC2* may be associated with enhanced resistance against *P. rapae* feeding. Hence, blocking this response would be beneficial to *P. rapae*. Addressing this question will be one of the challenges for future research.

As became evident from GeneChip data (Reymond *et al.*, 2000; 2004), damage as a result of feeding by herbivorous insect leads to water losses, which trigger the production of abscisic acid (ABA) and ABA-responsive gene expression. *AtMYC2* expression is also induced by ABA (Abe *et al.*, 1997; Anderson *et al.*, 2004), as it is by JA (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004), and *P. rapae* feeding (Chapter 4). As shown in Chapter 5, feeding by *P. rapae* also induced another drought- and ABA-responsive transcription factor, *AtMYB102*. GUS staining showed expression of *AtMYB102* at the feeding edges, and this transcription factor appears responsible for the up-regulation of a large set of genes. Over-expression of *AtMYB102* triggered expression of a large number of genes (150 out of the 6,000 studied), most of which are involved in cell wall modification (Chapter 5; Fig. 5). Such modifications might contribute to a defense response effective against *P. rapae* attack.

## **The network of induced defense signaling pathways: a working model**

Previous research and the research described in this thesis shed new light on the complexity of induced resistance signaling in Arabidopsis. Figure 1 provides a simplified working model, that helps to understand the functioning of the plant's induced defense response. Clearly, SA, JA, and ET are primary signals that upon attack are produced in a blend that can vary significantly in composition, timing, and amplitude. Hence, the signal signature of a given plant-attacker combination sets the scene for the defense response that is activated in the plant. ABA is emerging as another important regulator of induced resistance (Anderson *et al.*, 2004; Audenaert *et al.*, 2002; Mauch-Mani and Mauch, 2005; Ton *et al.*, 2005). Although these signal molecules are important primary signals in induced defense, additional regulatory mechanisms shape the final outcome



**Figure 1.** Working model of the signaling network that regulates induced defense responses in *Arabidopsis* upon pathogen infection, wounding, and feeding by *P. rapae* larvae.

of the resistance response. In this respect, cross-talk between defense signaling pathways provides a powerful regulatory potential. For instance, SA produced upon pathogen attack is transduced through NPR1, leading to the activation of SA-responsive genes, such as *PR-1*, and an elevated level of protection (SAR). Simultaneously, SA-activated NPR1 suppresses JA signaling (Spoel *et al.*, 2003), thereby prioritizing SA-inducible defenses over JA-inducible ones. Some necrotrophic pathogens, such as *A. brassicicola*, trigger the production of JA in the plant, resulting in the activation of JA-responsive genes, such as *PDF1.2* (Chapter 2, Fig. 3). However, other JA-responsive genes, such as *VSP2*, are not activated in this interaction, suggesting that *PDF1.2* and *VSP2* are part of different branches of the JA response. Indeed, Lorenzo *et al.* (2004) demonstrated that *ERF1* and *AtMYC2* are responsible for the differential activation of these two branches of the JA response. In contrast to infection by *A. brassicicola*, *P. rapae* feeding induced the expression of *VSP2* but not that of *PDF1.2*. Elicitors in the regurgitant of *P. rapae* appeared to affect the wound response by activating *AtMYC2*. As a result, the *ERF1* branch of the JA response that, among others, leads to *PDF1.2* gene expression is suppressed. ABA is required for *AtMYC2* expression, although it is not involved in the enhancement of the expression levels that are triggered by *P. rapae*. Wounding results in an ABA-dependent response of the plant that is activated to reduce damage caused by dehydration stress. This osmotic stress response was shown to activate the transcription factor gene *AtMYB102* that regulates the expression

of ABA-response genes, including a large number of genes that encode proteins involved in cell-wall strengthening (e.g. expansins). However, overexpression of this response does not seem to affect *P. rapae* performance.



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## Summary

Plants are sessile organisms that cannot flee from unfavorable conditions. Abiotic conditions, such as drought, cold, UV-irradiation, or flooding will severely influence plant fitness. In addition, plants can be attacked by a multitude of invaders, i.e. herbivorous insects or microbial pathogens. In order to cope with these threats plants have evolved sophisticated defensive mechanisms that limit damage caused by biotic or abiotic stress. In the work described in this thesis, we used the model plant species *Arabidopsis thaliana* to investigate molecular mechanisms involved in the ever ongoing battle between plants and their microbial and herbivorous enemies. In order to rapidly respond to pathogen or insect attack, plants possess a variety of inducible defense responses, which are initiated upon recognition of the attacker. The plant signaling molecules, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) have been shown to play an important regulatory role in these responses. Their levels increase in invaded tissue and trigger defense reactions that mount resistance at the site of interaction (local) or throughout the whole plant (systemic). Although there are exceptions, resistance against biotrophic microbial pathogens is largely dependent on the action of SA, while JA-dependent defense responses are often effective against necrotrophic pathogens and insects. The plant hormone ET has a modulating role in both SA- and JA-dependent defense responses, but by itself can also confer resistance to some invaders. Recent advances in defense signaling research revealed that SA-, JA-, or ET-dependent defense responses do act independently of each other, but interact in a more complex signal-transduction network. For instance, induction of SA-dependent responses is known to suppress JA-dependent responses. This so-called cross-talk between signal transduction cascades is thought to provide the plant with a powerful regulatory potential, which helps the plant to “decide” which defensive strategy to follow, depending on the type of attacker it is encountering. Yet, it may also allow attackers to manipulate plants for their own benefit by shutting down induced defenses through influences on the signaling network.

In order to investigate to what extent plants make use of the interplay between SA-, JA-, and ET-dependent defense signaling pathways, we first compared the response of Arabidopsis to attack by different microbial pathogens and herbivorous insects, i.e. the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, the fungal pathogen *Alternaria brassicicola*, tissue-chewing larvae of the cabbage butterfly (*Pieris rapae*), cell-piercing thrips (*Frankliniella occidentalis*), and phloem-sucking aphids (*Myzus persicae*). We monitored the levels of SA, JA, and ET over time for each Arabidopsis-attacker combination (typically 0–72 hr). Data in chapter 2 show that each Arabidopsis-attacker combination leads to the accumulation of a specific blend of these signaling molecules, called the “signal signature”. The signal signature of each interaction varied greatly in composition, amplitude, and timing, indicating that plants are highly flexible in their response to different invaders. In addition, the expression of all ~25,000 Arabidopsis genes was studied in response to each of the five attackers by using Affymetrix Arabidopsis whole-genome GeneChips. Analysis of global gene expression profiles demonstrated that the signal signature characteristic of each Arabidopsis-attacker combination is orchestrated into a surprisingly complex set of transcriptional alterations in which, in all cases, stress-related genes are over-represented. Comparison of the transcript profiles revealed that consistent changes induced by pathogens and insects with very different modes of attack can show considerable overlap. However, the majority of the induced changes in gene expression were attacker-specific. Notably, although *P. syringae*, *A. brassicicola*, *P. rapae* and *F. occidentalis* all stimulated JA biosynthesis and JA-responsive gene expression, the majority of the changes in JA-responsive gene expression were attacker-specific. Hence, defense signals such as JA play a primary role in the orchestration of the plant’s defense response, but other regulatory mechanisms, such as pathway cross-talk and additional attacker-induced signals, eventually shape the highly complex attacker-specific defense response.

Next, we investigated whether prior attack by one invader would influence the resistance against another attacker. To this end, we developed a bioassay in which plants were infested by JA-inducing larvae of the herbivore *P. rapae* and subsequently inoculated with various microbial pathogens. We hypothesized that herbivore feeding would lead to increased levels of JA and, thus, would induce resistance against microbial pathogens that are restricted by JA-dependent defense responses (e.g. *A. brassicicola*), while microbial pathogens arrested through SA-dependent defenses (e.g. turnip crinkle virus (TCV)) would not be affected. Larvae of *P. rapae* stimulated the production of JA and triggered a defense response that affected insect performance on systemic tissues. Although *A. brassicicola* is sensitive to JA-dependent defenses, herbivore-induced resistance was not effective against this

pathogen. To investigate the reason why *P. rapae*-induced defense was not effective against *A. brassicicola*, we analyzed the expression of *PDF1.2*, a JA-responsive marker gene for resistance to *A. brassicicola*. *PDF1.2* was activated upon mechanical damage but suppressed when wounding was inflicted by *P. rapae* feeding. Application of larval regurgitant to artificially wounded sites suppressed wound-induced *PDF1.2* expression as well, indicating that elicitors from *P. rapae* antagonize this JA-dependent defense response. This may explain the ineffectiveness of herbivore-induced resistance against *A. brassicicola*. Resistance against the biotrophic turnip crinkle virus (TCV) requires SA, but not JA and ET. Nevertheless, herbivore feeding strongly reduced TCV multiplication and TCV lesion formation, also in systemic tissues. Wounding alone was not effective, but application of regurgitant onto the wounds induced a similar level of protection. Analysis of SA-induced *PR-1* expression revealed that *P. rapae* feeding primes Arabidopsis leaves for augmented expression of SA-dependent defenses. Pharmacological experiments showed that ET acts synergistically on SA-induced *PR-1*, suggesting that the increased production of ET upon herbivore feeding sensitizes the tissue to respond faster to SA, thereby contributing to an enhanced defensive capacity towards pathogens, such as TCV, that trigger SA-dependent defenses upon infection. Hence, feeding by *P. rapae* triggers a surprisingly complex defense response that includes both synergistic and antagonistic effects on cross-talk between different signaling pathways that lead to resistance against microbial pathogens.

The observation that factors in the regurgitant of *P. rapae* suppress wound-induced expression of *PDF1.2* prompted us to investigate the molecular mechanism underlying this phenomenon. To investigate the mechanism by which *P. rapae* feeding suppresses *PDF1.2* expression, we studied the role of SA and abscisic acid (ABA), both of which have been implicated in antagonizing the JA-induced expression of *PDF1.2*. *P. rapae*-mediated suppression of *PDF1.2* was shown to be independent of SA for two reasons. Firstly, other JA-responsive genes, which were shown previously to be suppressed by SA were not affected by *P. rapae* feeding. Secondly, the regulatory protein NPR1, which is important in SA-mediated suppression of *PDF1.2*, is not required for *P. rapae*-mediated suppression of *PDF1.2*. However, the ABA biosynthesis mutant *aba2-1* showed a significantly increased *PDF1.2* expression upon feeding by *P. rapae*. Previously, ABA was shown to be an important regulator of AtMYC2, a transcription factor that activates specific JA-responsive genes (e.g. *VSP2* and *LOX2*), while suppressing other JA-responsive genes (e.g. *PDF1.2*). AtMYC2 was up-regulated in response to *P. rapae* feeding, but not upon mechanical damage. Like *aba2-1*, the AtMYC2 mutant *jin1-2* was also impaired in *P. rapae*-induced suppression of *PDF1.2* and showed high levels of *PDF1.2* expression upon insect feeding. Suppression of other wound-responsive, *P.*

*rapae*-suppressed genes, e.g. *ETHYLENE-RESPONSE FACTOR*, showed a strong *P. rapae*-induced expression pattern in *jin1-2*. Taken together, our results indicate that AtMYC2 is an important regulator of *P. rapae*-induced suppression of a specific branch of the JA-dependent host defense response. It is tempting to speculate that the specialist caterpillar, *P. rapae*, actively interferes with the host defense response and thereby makes it more suitable for infestation. On the other hand, the host might shut down unnecessary defense responses that do not contribute to the defense against this particular invader, in order to prioritize defenses that do affect caterpillar feeding.

Finally, the involvement of another transcription, AtMYB102, in resistance against *P. rapae* was investigated. This member of the MYB transcription factor family was previously shown to be induced upon wounding and osmotic stress. As both stresses occur during caterpillar attack, we hypothesized that AtMYB102 would also be involved in resistance against feeding *P. rapae* larvae. Indeed, independent experiments showed that *AtMYB102* is up-regulated upon caterpillar feeding. Histochemical analysis of an *AtMYB102*:*GUS* reporter line indicated that *AtMYB102* was expressed along the edges of the feeding sites. Knockout *myb102* mutants with a T-DNA insertion in the *AtMYB102* gene allowed the caterpillars to gain a 1.5-fold higher weight than caterpillars feeding from Col-0 wild-type plants. Moreover, approximately 50% of all larvae feeding on *myb102* plants had pupated within 14 days, whereas only 5% did on wild-type plants. These results indicate that MYB102 plays a role in the defense response of Arabidopsis to herbivore feeding.

In conclusion, we demonstrated that plants are highly flexible in recognizing different attackers and respond by inducing an attacker-specific signal signature and transcript profile. Although one can make predictions based on signal signature and transcript profile whether or not prior attack by one invader will affect the resistance against a subsequent attacker, we have shown that these predictions are not necessarily correct. In particular, specialized attackers might manipulate pathway cross-talk for their own benefit and thereby make the host plant more susceptible to subsequent infection by pathogenic micro-organisms or infestation by herbivorous insects. Hence, this research provided new insights into the complexity of the plant's response to harmful organisms. However, many intriguing questions remain on the continuing arms race between a host and its specialized attackers.

## Samenvatting

Planten staan voortdurend bloot aan stresscondities, zoals droogte, kou, UV-straling of overstroming. Naast abiotische stress hebben planten ook te maken met biotische stress veroorzaakt door een grote verscheidenheid aan microbiële ziekteverwekkers (pathogenen zoals schimmels, bacteriën, virussen en aaltjes) en herbivore insecten. Omdat planten niet kunnen weglopen voor deze ongunstige omstandigheden, hebben ze gedurende de evolutie effectieve afweermechanismen ontwikkeld, die schade als gevolg van abiotische of biotische stress tegengaan. In het onderzoek dat is beschreven in dit proefschrift is gebruik gemaakt van de modelplant *Arabidopsis thaliana* (zandraket) om de afweermechanismen van planten tegen pathogenen en insecten op moleculair niveau te bestuderen.

Als een plant wordt belaagd door een pathogeen of insect worden in het geïnfecteerde weefsel diverse afweermechanismen geactiveerd die erop gericht zijn aantasting en schade te beperken. Afhankelijk van het type belager is de plant in staat om verschillende afweermechanismen aan te schakelen. De signaalmoleculen salicylzuur (SA), jasmonzuur (JA) en ethyleen (ET) spelen daarbij een belangrijke rol. Na herkenning van een pathogeen of insect wordt de productie van één of meer van deze signaalmoleculen verhoogd waarna in het aangetaste plantenweefsel een specifieke afweerreactie wordt geïnduceerd.

Een verhoogde productie van SA wordt vaak in verband gebracht met geïnduceerde afweer tegen biotrofe pathogenen, die alleen voedsel kunnen onttrekken aan levende cellen. Inductie van de biosynthese van JA bevordert afweer tegen insecten en necrotrofe pathogenen, die het weefsel eerst doden alvorens zij daarin kunnen groeien. Het plantenhormoon ET speelt bij beide verdedigingsreacties een modulerende rol. Onderzoek naar de rol van SA, JA en ET heeft aangetoond dat de drie signaalmoleculen interacteren in een complex netwerk van signaal-transductieroutes die gezamenlijk bepalend zijn voor het afstemmen van de afweerreactie op het pathogeen of insect dat de plant belaagt. De interactie tussen de verschillende signaal-transductieroutes wordt

“cross-talk” genoemd. “Cross-talk” wordt veelal gezien als een mogelijkheid voor de plant om de meest ideale reactie te induceren die schade als gevolg van een bepaalde stressfactor kan minimaliseren. Gespecialiseerde aanvallers maken juist gebruik van deze “cross-talk” om de afweerreactie van de plant zodanig te manipuleren dat zij daarvan geen last ondervinden. Daarmee creëren ze voor zichzelf een leefmilieu dat niet gespecialiseerde aanvalleur niet kunnen benutten.

Om inzicht te krijgen in de “cross-talk” tussen de verschillende signaal-moleculen is *Arabidopsis* blootgesteld aan vijf typen belagers: de bacterie *Pseudomonas syringae* pv. *tomato*, de schimmel *Alternaria brassicicola*, en de insecten *Pieris rapae* (larven van het kleine koolwitje), *Frankliniella occidentalis* (thrips) en *Myzus persicae* (groene perzikluis) (hoofdstuk 2). Analyse van de hoeveelheden SA, JA en ET in aangetaste bladeren liet zien dat iedere plant-belager combinatie leidt tot productie van een specifieke mix van deze signaalmoleculen. Deze zogenaamde “signal signature” verschilt in zowel in samenstellingen, relatieve hoeveelheden als in de snelheid waarmee de toenames optreden. De “signal signature” omvat derhalve een groot regulerend potentieel voor de afstemming van de afweerreactie.

Om te onderzoeken hoe de “signal signature” wordt vertaald in het activeren van specifieke afweerreacties, werd de expressie van alle ~23.000 genen van *Arabidopsis* bestudeerd. Hiervoor werd gebruik gemaakt van zogenaamde genenchips (*Arabidopsis* whole-genome Affymetrix ATH1 GeneChips). Analyse van de geïnduceerde genexpressiepatronen liet zien dat iedere “signal signature” in de plant wordt vertaald in een specifiek en uiterst complex profiel van genexpressie. Ondanks het feit dat sommige *Arabidopsis*-belager interacties een vergelijkbare “signal signature” hadden, was de overlap in geïnduceerde genexpressie toch gering. Bijvoorbeeld: *P. syringae*, *A. brassicicola*, *P. rapae* en *F. occidentalis* induceerden alle vier de productie van JA en activeerden hoofdzakelijk genen die door JA worden gereguleerd. Echter, de overlap tussen de door JA gereguleerde genen in de vier *Arabidopsis*-belager combinaties was zeer klein. Dit duidt erop dat behalve de primaire signaalmoleculen SA, JA en ET andere regulatiemechanismen actief moeten zijn die de afstemming van de afweerreactie van de plant op een bepaalde belager beïnvloeden.

Vervolgens stelden wij onszelf de vraag in hoeverre een aanval door een bepaalde belager de afweerreactie tegen een volgende belager zou beïnvloeden. Hiervoor hebben we een biotoets opgezet waarin in *Arabidopsis* resistentie tegen insectenvraat werd geïnduceerd door larven van *P. rapae* om vervolgens de effectiviteit van deze resistentie tegen diverse microbiële pathogenen te kunnen testen (hoofdstuk 3). Een eerdere veronderstelling was dat geïnduceerde JA-afhankelijke afweer tegen larven van het koolwitje ook een effectieve resistentie zou opleveren tegen pathogenen die gevoelig zijn voor afweermechanismen

die worden gereguleerd door JA (zoals *A. brassicicola*). Ten tweede werd verondersteld dat de door *P. rapae* geïnduceerde resistentie niet effectief zou zijn tegen pathogenen die niet gevoelig zijn voor afweerreacties die gereguleerd worden door JA, maar die wel geïnduceerd worden door SA (zoals Turnip crinkle virus (TCV)). Echter, planten die waren geïnduceerd door *P. rapae* hadden een verhoogde resistentie tegen TCV. Vervolgexperimenten lieten zien dat ET, dat in Arabidopsis bladeren wordt geproduceerd in reactie op vraat door *P. rapae*, het bladweefsel verhoogd gevoelig maakt voor SA, waardoor de plant beter in staat is om op een SA-inducerende belager te reageren. In tegenstelling tot de verwachting induceerde insectenvraat geen resistentie tegen *A. brassicicola*. De in hoofdstuk 3 en 4 beschreven resultaten suggereren dat vraat door *P. rapae* een specifieke set van door JA gereguleerde genen onderdrukt. Deze set genen omvat o.a. *PDF1.2*, een belangrijk marker-gen dat codeert voor een eiwit met antischimmel activiteit en geassocieerd wordt met verhoogde resistentie tegen *A. brassicicola*.

In hoofdstuk 4 wordt ingegaan op het moleculaire mechanisme van deze onderdrukking van de afweer door *P. rapae*. Allereerst bleek niet alleen vraat door *P. rapae* larven, maar ook het aanbrengen van “spuug” (maaginhoud) van deze rupsen na verwonding van het blad de expressie van het *PDF1.2* gen te onderdrukken. Zowel SA als het hormoon abscisinezuur (ABA) hebben een antagonistische werking op de JA-respons in Arabidopsis. Daarom is de rol van deze beide signaalmoleculen bij de door *P. rapae* geïnduceerde remming van de expressie van *PDF1.2* onderzocht. SA bleek geen rol te spelen omdat door SA onderdrukte, zoals JA geactiveerde genen (*VSP2* en *LOX2*) wel werden geïnduceerd na rupsenvraat. Bovendien werden dezelfde resultaten verkregen in de *npr1* mutant van Arabidopsis, die gestoord is in deze “cross-talk” tussen SA en JA. De door *P. rapae* geïnduceerde onderdrukking van *PDF1.2* was echter niet meer aanwezig in de ABA biosynthese mutant *aba2-1*. ABA is een belangrijke positieve regulator van de expressie van het *AtMYC2* gen dat codeert voor een transcriptiefactor die een rol speelt bij de regulatie van de JA respons. Het bleek dat het *AtMYC2* gen wordt geïnduceerd door factoren in het spuug van *P. rapae*, en dat de *AtMYC2* transcriptiefactor essentieel is voor de onderdrukking van de set van door JA geïnduceerde genen die *PDF1.2* omvat. Deze resultaten wijzen op een actieve onderdrukking van de verdediging van de plant door *P. rapae*.

Tenslotte hebben we de rol bij de resistentie tegen *P. rapae* bestudeerd van *AtMYB102*, een door verwonding en osmotische stress geïnduceerde transcriptiefactor (hoofdstuk 5). Expressie van *AtMYB102* werd geactiveerd na vraat door larven van *P. rapae*. De hoeveelheid mRNA nam sterk toe in door rupsen beschadigde bladeren, met name langs de vraatranden. *P. rapae* larven ontwikkelden zich sneller op transgene planten die geen functioneel

AtMYB102 eiwit meer kunnen aanmaken (*myb102*) dan op wild-type planten. Deze resultaten duiden op een bijdrage van de transcriptie factor AtMYB102 aan de resistentie van *Arabidopsis* tegen vraat door *P. rapae*. Een vergelijking van de genexpressie in wild-type planten en planten die AtMYB102 tot overexpressie brengen duidde in de richting van veranderingen in de celwanden als gevolg van overexpressie van AtMYB102. Deze veranderingen zouden kunnen bijdragen aan de afweerreactie tegen vraat door larven van *P. rapae*.

Samenvattend kunnen we concluderen dat planten uiterst flexibel blijken in het herkennen van hun belagers. Na herkenning reageert de plant met de inductie van een belager-specifieke “signal signature” en treedt activering op van een specifieke set van genen die nodig zijn om het meest adequate verdedigingsmechanisme in werking te zetten. Daarnaast is duidelijk geworden dat voorspellingen die gebaseerd zijn op de plant “signal signature” en, over het effect van een eerste aanval op de resistentie tegen een volgende belager niet altijd correct zijn. Ook blijken gespecialiseerde belagers bestaande signaal transductie routes te manipuleren. Daarbij verstoren zij “cross-talk” om effectieve verdedigingsmechanismen in de plant te onderdrukken. De continue wapenwedloop tussen waardplant en gespecialiseerde belagers blijft daarom een intrigerend onderzoeksveld.

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## Dankwoord

Tja, dat is ook een onderdeel van het proefschrift en moet dus ook netjes afgewerkt worden. Het is een mogelijkheid om mensen te bedanken die om een of andere reden een bijdrage hebben geleverd aan het tot stand komen van dit boekje. Voor mij een reden om familie, vrienden en collega's te bedanken. Maar waar begin je dan?

Beginnen bij het begin, dus bij mijn ouders: jullie hebben altijd een, soms misschien onzichtbare, rol gespeeld gedurende mijn biologie studie en tijdens mijn AiO-periode. Bedankt voor alle steun. Natuurlijk wil ik ook Inge en haar familie bedanken voor al hun enthousiasme en steun.

Steun en afleiding kwam ook van vrienden. En inderdaad dat zijn (vaak) korfballers, want gedurende de 4 jaar dat ik in Utrecht mijn promotie onderzoek gedaan heb, waren er gemiddeld 4 avonden en een zaterdag afleiding te vinden in dat spelletje dat mijn collega's maar niet als sport willen zien. Daarnaast moest ik om de week op zondag ook nog eens naar de plaatselijke FC om samen met Maarten van ons prachtige uitzicht te genieten (gold zeker niet altijd voor het vertoonde spel, maar toch). Of lasagne eten met de bolle (Gert-Jan, mijn broertje, die allang niet zo klein meer is natuurlijk) om na studio-sport (dit was voor het talpa-tijdperk) met z'n vieren nog een DVD-tje te kijken. Ook kan ik hier de diverse bezoeken van Mike Shane aan Nederland niet vergeten. Iedere keer wist ie ons weer te verrassen met een stop op weg naar "the motherland", waarna ik vervolgens toerist in eigen land moest spelen.

Tot slot in dit rijtje de biologie makers. Eens in het halfjaar komen ze bijeen om even weg te zijn van thuis. Onder het genot van een warme chocolademelk of een biertje wordt dan geklaverjast tot ze erbij neervallen. Eelco, Werner, Roderick en ikzelf hebben ons nog steeds niet ingeschreven voor dat bejaardenhuis waar we het al jaren over hebben (wie regelt dat nu eens??).

Graag wil ik even aandacht besteden aan de collega's in Utrecht. Dit is echt een gezellig zootje ongeregeld. Zeer sociaal ingesteld en altijd daar om de ander op te vrolijken. Het feit dat iedereen een bijdrage levert aan deze

gezelligheid betekent dat het nog wel lang goed zal blijven gaan bij Fyto. Als ik aan onze koffie, lunch, of thee pauzes denk, verschijnt er glimlach op mijn gezicht. En dan denk ik bijvoorbeeld aan het verhaal van de komkommerkweker met zijn witte schimmel.

Dat “we” hier met een hechte groep te maken hebben blijkt wel uit de belevenissen die vanuit het door een orkaan geteisterd Cancun komen. Ik denk dat de vaste staf trots mag zijn op de sfeer die er in Utrecht heerst! Ik mis jullie!

Okay, nu het serieuze gedeelte: Corné, bedankt voor je tomeloze inzet! En je, zoals je graag zelf zegt, “briljante ingevingen”. Ondanks dat je zelf misschien nog wel meer tijd wil vrijmaken voor het onderzoek van een ieder die je begeleidt, denk ik dat je het uitstekend hebt gedaan! Vooral de laatste maanden waren best hectisch, vooral omdat ik ineens had besloten dat ik per 1 september naar de VS wilde voor een post-doc en dus moest alles nog eerder klaar zijn dan vooraf misschien gepland. Je eerlijke commentaar op de door mij geschreven hoofdstukken was soms overduidelijk tegen middennacht geschreven, hetgeen soms ook een klein beetje doorschemerde in de track-changes optie in word (gelukkig was jij altijd de eerste die dat er dan ook meteen bij wist te zetten). Ik hoop dat je in iets rustiger vaarwater terecht bent gekomen na mijn vertrek en je inaugurele rede (nog van harte!). Klein advies: misschien toch wat taken afstoten?

Kees, bedankt voor de ondersteuning bij het schrijven van mijn proefschrift. Je uitstekende commentaar en suggesties bij de introductie en discussie hebben deze hoofdstukken tot een mooi geheel gemaakt.

Marcel, de samenwerking tussen Fyto en Ento is in mijn ogen zeer vruchtbaar geweest en het zal zeker de moeite waard zijn dit te continueren. Bedankt voor het aanleveren van de gewenste insecten en de waardevolle discussies die we hebben gehad.

Vief, twinnie, het was leuk om min of meer tegelijkertijd te starten met ons promotie traject. Ik heb genoten van het werk en de gezelligheid die ons gezamenlijke experimenten met zich mee brachten. Ik hoop dat je proefschrift er ook snel zal liggen, en denk dat je dan heel trots mag zijn!

Daarnaast waren er nog twee master studenten die ieder hun bijdrage hebben geleverd aan mijn onderzoek. Maaïke en Christiaan, bedankt voor jullie inzet. Bovendien mag ik Wendy niet vergeten te noemen: gedurende de tijd dat zij haar stage voor de HLO afwerkte hebben we een enorme hoeveelheid werk weten te verzetten. Mede door jouw praktische vaardigheden konden we iedere week minimaal één grote bio-toets afronden, hetgeen geresulteerd heeft in een hoofdstuk (hoofdstuk 3) dat klaar is voor publicatie en waar jouw naam de lijst met auteurs kleur zal geven. Bedankt voor je inzet en de bijdrage die

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Tot slot, nog even aandacht voor de onderzoeksschool waar ik als AiO deel van uit maakte. Ik denk dat het onderwijs en onderzoek binnen EPS van een goede kwaliteit is, hetgeen voornamelijk het werk is van de individuele leerstoelgroepen, maar ook van EPS dat als een uitstekend platform dient.



## Curriculum vitae

Martin de Vos werd geboren op 11 december 1977 te Amersfoort. In 1996 behaalde hij zijn VWO diploma aan het Nieuwe Eemland College te Amersfoort. In datzelfde jaar werd aangevangen met de studie Biologie aan de Universiteit Utrecht. Tijdens de doctoraalfase werden twee onderzoeksstages vervuld: allereerst bij de leerstoelgroep Fytopathologie waar onder begeleiding van Dr. Jurriaan Ton werd gewerkt aan “Characterization of Arabidopsis enhanced disease susceptibility mutants that are affected in systemically induced resistance”. Om daarna de opleiding te vervolgen in het buitenland met een stage aan de University of Western Australia, Perth, ditmaal onder begeleiding van Dr. Michael Shane en Prof. Dr. Hans Lambers met als titel: “Regulation and functioning of cluster roots in response to phosphorus-limiting conditions in crop and Australian native plant species”. Aldaar werd ook een literatuurstudie getiteld “Early signaling in the establishment of mycorrhizal symbiosis between AM fungi and host plants” voltooid. In augustus 2001 studeerde hij af, om vervolgens direct als AIO bij de leerstoelgroep Fytopathologie van de Universiteit Utrecht aan de slag te gaan. Daar werd onder begeleiding van Prof. Dr. Ir. Corné Pieterse en Prof. Dr. Ir. L.C. Van Loon, en in samenwerking met Prof. Dr. Marcel Dicke (Entomologie, Universiteit Wageningen), tot augustus 2005 het onderzoek uitgevoerd dat in dit proefschrift is beschreven.

