

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

Martin De Vos<sup>1</sup>, Marcel Dicke<sup>2</sup>, L.C. Van Loon<sup>1</sup> and Corné M.J. Pieterse<sup>1</sup>

- 1 Graduate School Experimental Plant Sciences, Section Phytopathology, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 800.84, 3508 TB Utrecht, The Netherlands
- 2 Graduate School Experimental Plant Sciences, Laboratory of Entomology, Wageningen University, P.O. Box 8031, 6700 EH Wageningen, The Netherlands

**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.

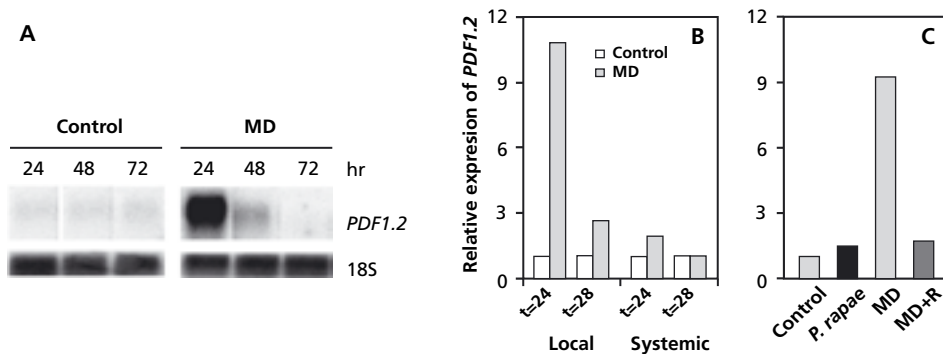
Previously, we tested the effectiveness of *P. rapae*-induced resistance against different microbial pathogens (Chapter 3). *P. rapae* feeding induces JA biosynthesis in damaged tissue (De Vos *et al.*, 2005). Although the necrotrophic fungus *A. brassicicola* is sensitive to JA-dependent defenses, *P. 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

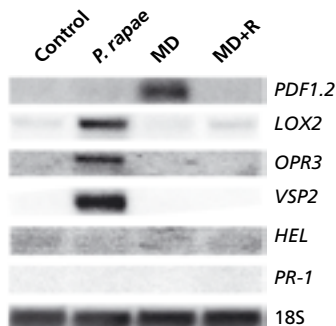
expression of the well-characterized JA-responsive genes *LIPOXYGENASE2* (*LOX2*) and *12-OXOPHYTODIENOATE REDUCTASE3* (*OPR3*), which encode key enzymes in the JA biosynthetic pathway (Bell *et al.*, 1995 and Schaller *et al.*, 2000, respectively), and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*). Arabidopsis 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).

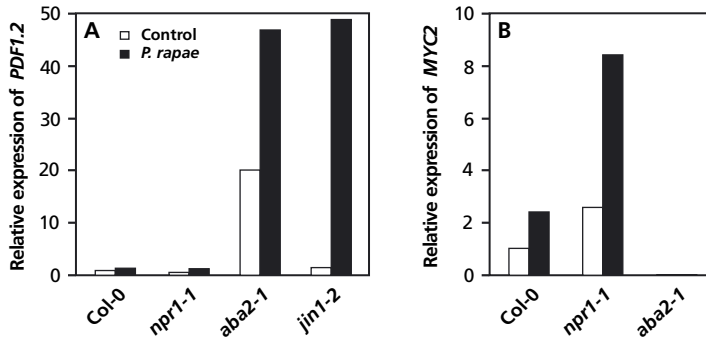
### **Role for ABA in herbivore-mediated suppression of *PDF1.2***

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.



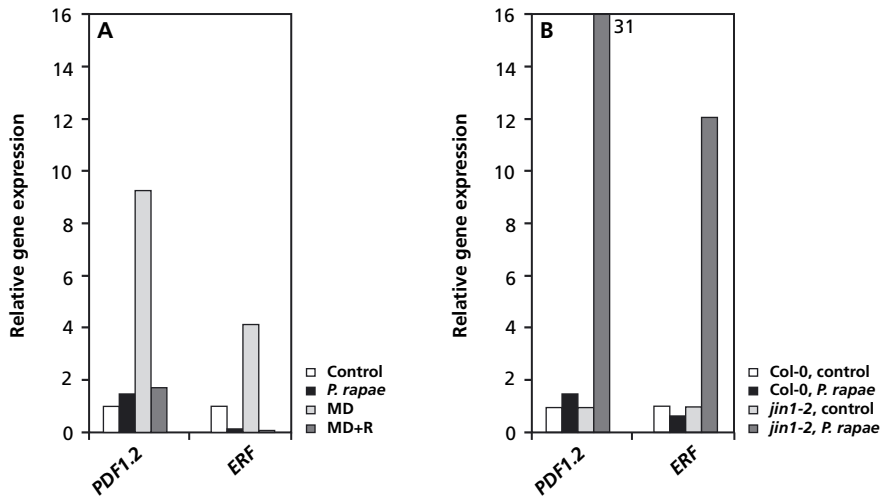
**Figure 3.** Expression patterns of *PDF1.2* and *AtMYC2* in wild-type Col-0, and mutant *npr1-1*, *aba2-1*, and *jin1-2* plants 24 hr upon *P. rapae* feeding.

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 *jin1-2* 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 *jin1-2* 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.

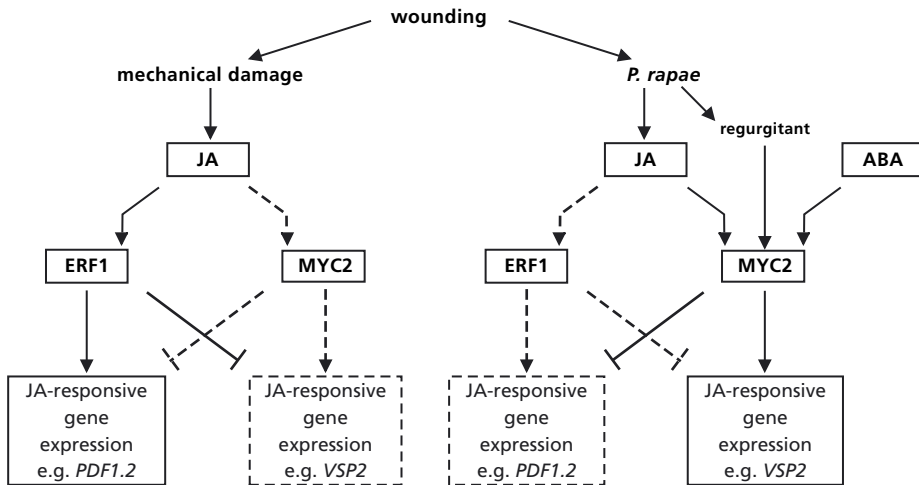
Besides SA, ABA has been demonstrated to suppress JA-responsive gene expression. Anderson *et al.* (2004) demonstrated that exogenous application of ABA suppressed both basal and JA-activated transcription of *PDF1.2*. Moreover, ABA deficiency as conditioned by mutations in the ABA biosynthesis genes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

## Acknowledgements

The authors acknowledge the use of microarray data produced by the AtGenExpress project, which was coordinated by Lutz Nover (Frankfurt), Thomas Altmann (Potsdam) and Detlef Weigel (Tübingen), and supported by funds from the DFG. The data set on mechanical wounding was produced by Stefan Weinl, Cecilia D'Angelo, Dragica Blazevic, Jörg Kudla (Universität Münster) and by Joachim Kilian, Jakub Horak, Dierk Wanke and Klaus Harter (Universität Tübingen). Furthermore, we acknowledge Ruth Joosten, Wendy van Zaanen, and Christiaan de Boer for technical assistance, and Leo Koopman, Frans van Aggelen and André Gidding for insect rearing. This research was supported, in part, by grants 865.03.002 and 865.04.002 of the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organization of Scientific Research (NWO).

**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).

