

# 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-EXPRESSION 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

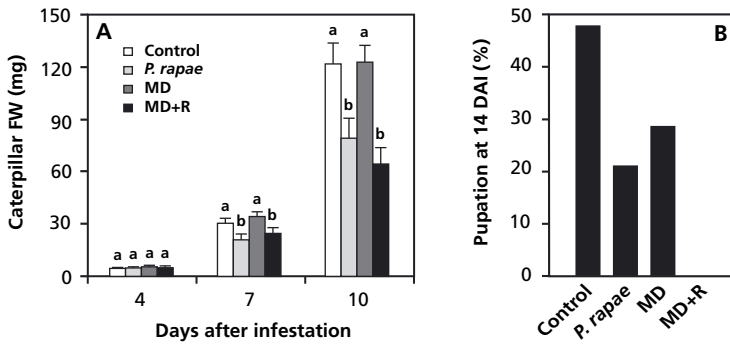
insect-induced signals into specific defense responses, we recently monitored the dynamics of SA, JA, and ET signaling in Arabidopsis after attack by a set of microbial pathogens and herbivorous insects with different modes of attack (De Vos *et al.*, 2005). Of these, the tissue-chewing caterpillar of 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-



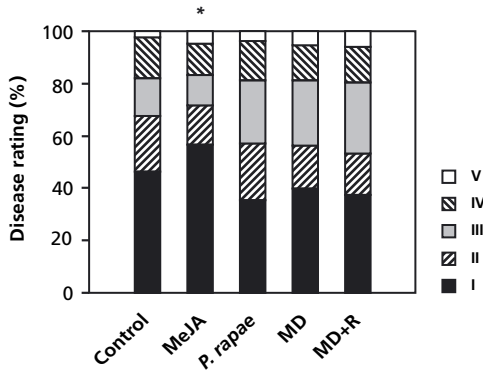
**Figure 1.** Effect of herbivore-induced resistance on *P. rapae* performance

(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.

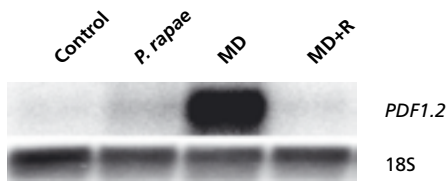


**Figure 2.** Effectiveness of herbivore-induced resistance against *A. brassicicola*

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.

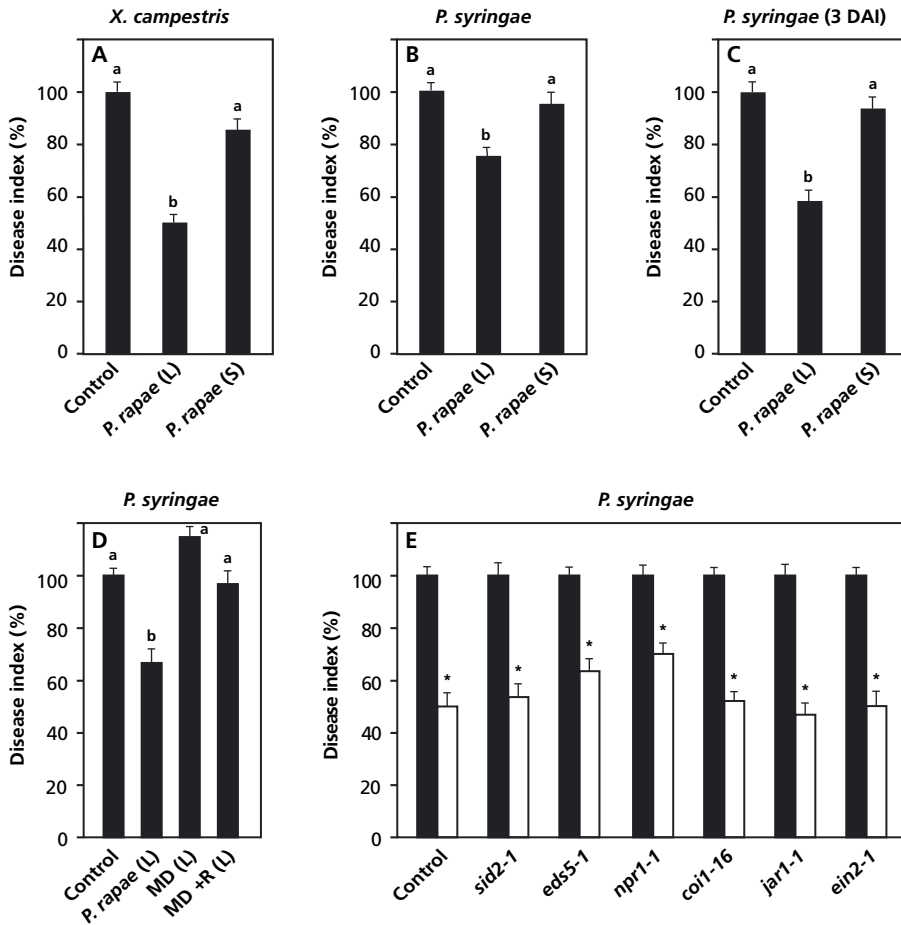
Because *P. rapae*-induced plants were challenge inoculated with the bacterial pathogens immediately after removal of the caterpillars, the time between induction and expression of resistance may have been too short to mount an effective systemic effect. To clarify this point, *P. rapae*-induced plants 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*.

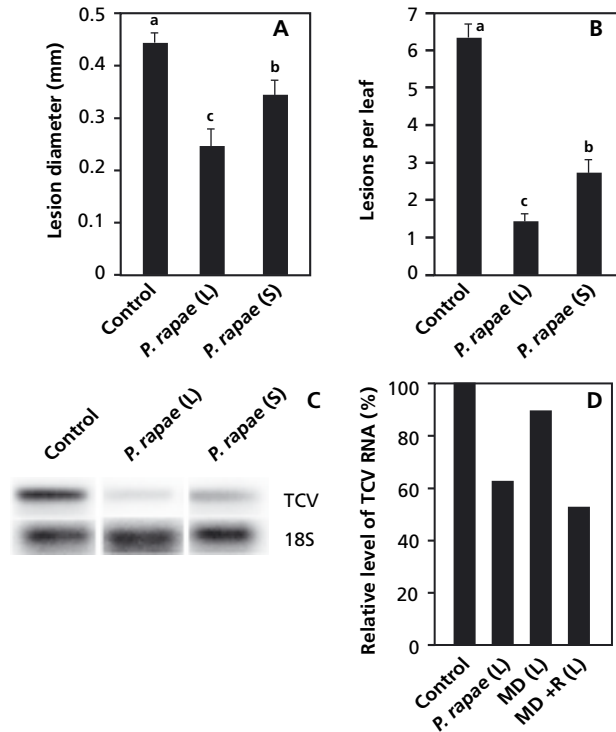
**E.** *P. rapae*-induced resistance against *P. syringae* pv. *tomato* in Arabidopsis defense signaling mutants. The absolute proportions of diseased leaves of uninduced control plants were 41% (A), 60% (B), 62% (C), 79% (D) and 72% (Col-0), 85% (*sid2-1*), 75% (*eds5-1*), 77% (*npr1-1*), 44% (*coi1-16*), 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 ( $0.1 \mu\text{g} \cdot \mu\text{L}^{-1}$ ) 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.

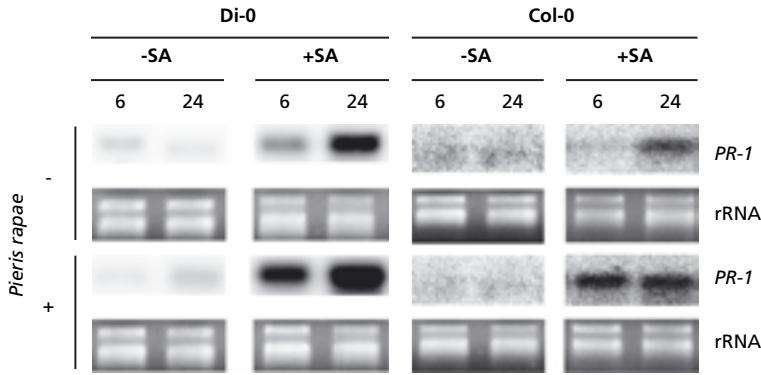
**B.** Local and systemic effects of *P. rapae*-induced resistance on the number of lesions per leaf. The values presented are means ( $\pm$ SE) from 15 plants that received the same treatment.

**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



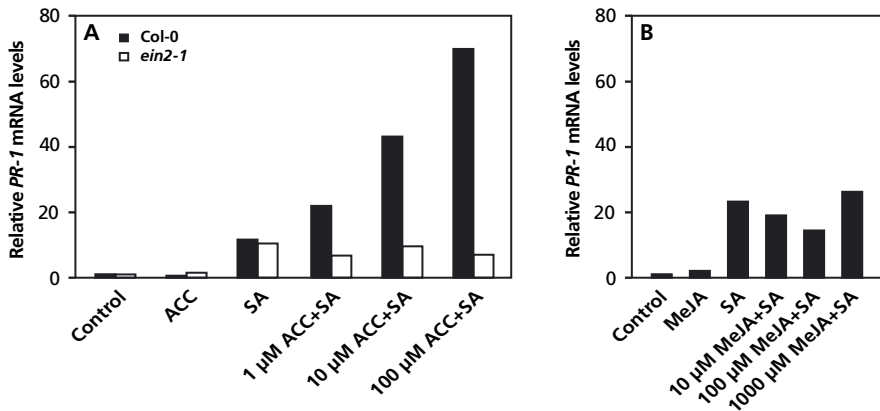
**Figure 6.** *P. rapae*-induced priming of SA-induced *PR-1* gene expression

*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

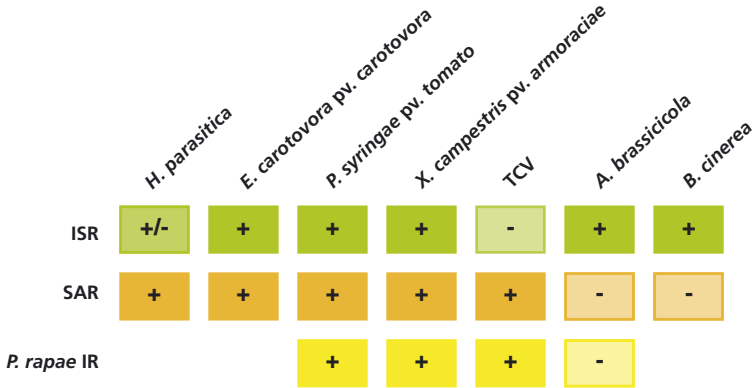
Prior infestation with *P. rapae* inhibited multiplication of TCV and significantly reduced the size and number of TCV lesions (Fig. 5). The effect of *P. rapae*-induced resistance against TCV was apparent not only locally in herbivore-damaged tissue, but also systemically in undamaged leaves of infested 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).



**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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