

General discussion

Plants under attack

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

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

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

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

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

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

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

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

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

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

Attacker-specific transcriptome changes in *Arabidopsis*

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

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

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

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

Cross-resistance: it is not that obvious

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

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

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

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

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

Clever attackers: making pathway cross-talk your advantage

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

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

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

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

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

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

The network of induced defense signaling pathways: a working model

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

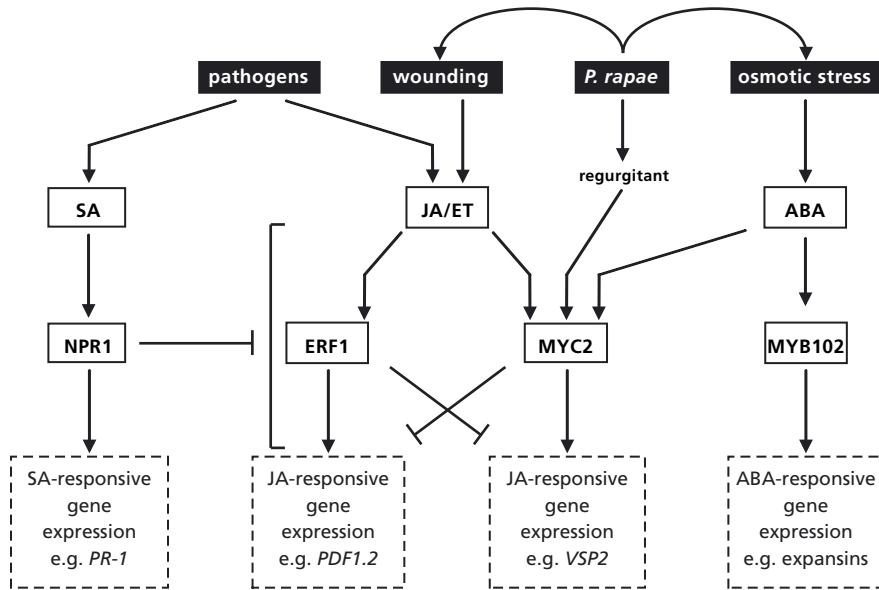


Figure 1. Working model of the signaling network that regulates induced defense responses in *Arabidopsis* upon pathogen infection, wounding, and feeding by *P. rapae* larvae.

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

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

