Chapter 4
Signalling cascades involved in induced resistance

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4.1 Introduction

Plant innate immunity is based on a surprisingly complex response that is highly flexible in its capacity to recognize and counteract different invaders. To effectively combat invasion by microbial pathogens and herbivorous insects, plants make use of pre-existing physical and chemical barriers, as well as inducible defence mechanisms that become activated upon attack (see Chapter 6). Apart from reacting locally, plants can mount a systemic response, establishing an enhanced defensive capacity in parts distant from the site of primary attack. This systemically induced response protects the plant against subsequent invaders. Several biologically induced, systemic defence responses have been characterized in detail, such as systemic acquired resistance (SAR), which is triggered by pathogens causing limited infection, such as hypersensitive necrosis (Durrant & Dong, 2004), rhizobacteria induced systemic resistance (ISR), which is activated upon colonization of roots by selected strains of non-pathogenic rhizobacteria (Van Loon *et al.*, 1998; Pieterse *et al.*, 2003; see also Chapter 8), and wound induced defence, which is typically elicited upon tissue damage, such as caused by insect feeding (Kessler & Baldwin, 2002; Howe, 2005; see also Chapter 5).

Although different types of induced resistance are at least partially controlled by distinct signalling pathways, they all share the characteristic that they have broad spectrum effectiveness. In many cases, this enhanced defensive capacity cannot be attributed to direct activation of defence related genes. Instead, the broad spectrum protection is commonly based on a faster and stronger activation of basal defence mechanisms when an induced plant is exposed to either microbial pathogens or herbivorous insects. It is therefore hypothesized that the broad spectrum characteristic of induced resistance is largely based on this conditioning of the tissue to react more effectively to a stress condition. By analogy with a phenotypically similar phenomenon in animals and humans, this enhanced capacity to express basal defence mechanisms is called 'priming' (Conrath *et al.*, 2002).

The plant hormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are major regulators of induced resistance (Pieterse & Van Loon, 1999; Glazebrook, 2001; Thomma *et al.*, 2001). Plants respond with the production of a specific blend of these alarm signals upon pathogen or insect attack. The production of these signals varies greatly in quantity, composition and timing, and results in the activation of differential

sets of defence related genes that eventually determine the nature of the defence response against the attacker encountered (Reymond & Farmer, 1998; Rojo *et al.*, 2003; De Vos *et al.*, 2005). Global expression profiling of various Arabidopsis-attacker interactions revealed substantial crosstalk between SA-, JA- and ET-dependent defence pathways (Glazebrook *et al.*, 2003; De Vos *et al.*, 2005). Cross-communication between these pathways provides a powerful regulatory potential that allows the plant to fine-tune its defence responses. Other plant hormones, such as abscisic acid (ABA), brassinosteroids and auxins have been reported to also play a role in induced defence against pathogens, but their significance is understood less well (Jameson, 2000; Audenaert *et al.*, 2002a; Krishna, 2003; Nakashita *et al.*, 2003; Thaler & Bostock, 2004; Ton & Mauch-Mani, 2004; Mauch-Mani & Mauch, 2005; Ton *et al.*, 2005).

In this chapter, we aim to review the current status of induced disease resistance signalling research. We will focus on the roles of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) in the signalling cascades involved in the different types of induced resistance. In addition, we will cover two emerging new topics in induced resistance research: pathway crosstalk and priming.

4.2 SA, JA and ET: important signals in primary defence

Apart from their roles in plant development, SA, JA and ET have repeatedly been implicated in the regulation of primary defence responses. In many cases, infection by microbial pathogens and attack by herbivorous insects is associated with enhanced production of these hormones and a concomitant activation of distinct sets of defence related genes (Maleck *et al.*, 2000; Schenk *et al.*, 2000; Reymond *et al.*, 2004; De Vos *et al.*, 2005). Moreover, exogenous application of these compounds often results in an enhanced level of resistance (Van Wees *et al.*, 1999). Depending on the host – pathogen interaction, JA, SA, and ET appear to be differentially involved in basal resistance. It has been proposed that the defence signalling pathways that are induced are influenced by the mode of attack of the pathogen, i.e. whether it requires living plant cells (biotrophs) or kills host cells and feeds on the dead tissue (necrotrophs) (Parbery, 1996; Glazebrook, 2005). SA-dependent defence responses are usually associated with a form of programmed cell death known as the hypersensitive response. This response can restrict the growth of biotrophic pathogens by killing the infected cells. In fact, this type of defence is effective against a wide range of biotrophs, but usually fails to protect against, or can even be beneficial for, necrotrophic pathogens (Govrin & Levine, 2000; Thomma *et al.*, 2001). JA-dependent defence responses, which are not associated with cell death, are generally considered to provide an alternative defence against necrotrophs (McDowell & Dangl, 2000). Compelling evidence for the role of SA, JA and ET came from recent genetic analyses of plant mutants and transgenics that are affected in the biosynthesis or perception of these compounds.

4.2.1 SA

A central role for SA became apparent with the use of NahG transformants. NahG plants constitutively express the bacterial *NahG* gene, encoding salicylate hydroxylase, which converts SA into inactive catechol. Tobacco and *Arabidopsis thaliana* NahG plants show enhanced disease susceptibility to a broad range of oomycete, fungal, bacterial and viral

pathogens (Delaney *et al.*, 1994; Kachroo *et al.*, 2000). Genetic screens in Arabidopsis to unravel plant defence pathways have identified recessive mutants affected in SA signalling that also show enhanced susceptibility to pathogen infection. For instance, the *sid1*, *sid2* and *pad4* mutants are defective in SA accumulation in response to pathogen infection. As a result, these mutants display enhanced susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and the oomycete pathogen *Hyaloperonospora parasitica* (Zhou *et al.*, 1998; Nawrath & Métraux, 1999; Wildermuth *et al.*, 2001), confirming the importance of SA in basal resistance against these different types of pathogens.

4.2.2 JA

JA was similarly demonstrated to play a role in basal resistance. For example, both the *jar1* mutant, with reduced sensitivity to methyl jasmonate (MeJA), and the *fad3fad7fad8* triple mutant, which is defective in JA biosynthesis, exhibit susceptibility to normally non-pathogenic soil borne oomycetes of the genus *Pythium* (Staswick *et al.*, 1998; Vijayan *et al.*, 1998). In another study, mutant *fad3fad7fad8* showed extremely high mortality from attack by larvae of the common saprophagous fungal gnat, *Bradysia impatiens* (McConn *et al.*, 1997), demonstrating an important role of JA in primary defence against herbivorous insects. Recently, increased susceptibility of *jar1* to *Fusarium oxysporum* (Berrocal-Lobo & Molina, 2004) and impairment of induced resistance against cucumber mosaic virus in *fad3fad7fad8* mutants have been reported (Ryu *et al.*, 2004). The JA insensitive mutant *coi1* shows enhanced susceptibility to the bacterial leaf pathogen *Erwinia carotovora* (Norman-Setterblad *et al.*, 2000) and the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* (Thomma *et al.*, 1998). Conversely, overexpression of a JA carboxyl methyl transferase increased endogenous levels of MeJA and resulted in a higher resistance to *B. cinerea* (Seo *et al.*, 2001). Moreover, constitutive activation of the JA signalling pathway in the Arabidopsis mutant *cev1* resulted in enhanced resistance to *P. syringae* and the mildew fungi *Erysiphe cichoracearum, Erysiphe orontii,* and *Oidium lycopersicum* (Ellis *et al.*, 2002). All these examples clearly point to a role of JA in resistance against pathogens with diverse lifestyles, challenging the general notion that JA-dependent defence responses are predominantly effective against necrotrophic pathogens.

4.2.3 ET

The role of ET in plant resistance seems more ambiguous (Van Loon *et al.*, 2006). In some cases, ET is involved in disease resistance, whereas in other cases it is associated with symptom development. For instance, several ET insensitive mutants of Arabidopsis have been reported to exhibit enhanced disease susceptibility to *B. cinerea* (Thomma *et al.,* 1999), *Pst* DC3000 (Pieterse *et al.*, 1998) and *E. carotovora* (Norman-Setterblad *et al.*, 2000), indicating that ET-dependent defences contribute to basal resistance against these pathogens. A similar phenomenon was observed in soybean mutants with reduced sensitivity to ET, which developed more severe symptoms in response to infection by the fungal pathogens *Septoria glycines* and *Rhizoctonia solani* (Hoffman *et al.*, 1999). In addition, Knoester *et al.* (1998) reported that ET insensitive tobacco transformed with the mutant ET receptor gene *etr1-1* from Arabidopsis displayed susceptibility to the normally non-pathogenic oomycete *Pythium sylvaticum.* Thus, ET plays a role in non-host resistance

as well. In other cases, reduced ET sensitivity was associated with tolerance. For instance, ET insensitive tomato genotypes allowed wild-type levels of growth of virulent *Pst* DC3000 and *Xanthomonas campestris* pv. *vesicatoria*, but developed less severe symptoms of disease (Lund *et al.*, 1998; Ciardi *et al.*, 2000). A similar phenomenon was observed in the Arabidopsis ET insensitive *ein2* mutant, which displayed increased tolerance to virulent strains of both *Pst* DC3000 and *X. campestris* pv. *campestris* (Bent *et al.*, 1992). In addition, soybean mutants with reduced sensitivity to ET developed similar or less severe disease symptoms in response to the bacterial pathogen *P. syringae* pv. *glycinea* and the oomycete *Phytophthora sojae* (Hoffman *et al.*, 1999). In these interactions, ET is primarily involved in symptom development, rather than disease resistance.

4.3 SA, JA and ET: important signals in induced disease resistance

Upon primary infection or insect attack, plants develop enhanced resistance against subsequent invaders. A classic example of such a systemically induced resistance is activated after primary infection with a necrotizing pathogen, rendering distant, uninfected plant parts more resistant towards a broad spectrum of virulent pathogens, including viruses, bacteria and fungi (Kuć, 1982; see also Chapter 1). This form of induced resistance is often referred to as systemic acquired resistance (SAR, Ross, 1961) and has been demonstrated in many plant–pathogen interactions (Ryals *et al.*, 1996; Sticher *et al.*, 1997). Pathogen induced SAR is typically characterized by a restriction of pathogen growth and a suppression of disease symptom development compared to non-induced plants infected by the same pathogen (Hammerschmidt, 1999). Another, phenotypically similar form of induced resistance is rhizobacteria induced systemic resistance (ISR), which is activated upon colonization of plant roots by selected strains of non-pathogenic rhizobacteria (Van Loon *et al.*, 1998; Chapter 8). Although the terms SAR and ISR are taken to be synonymous (Hammerschmidt *et al.*, 2001), for convenience we distinguish between pathogen- and rhizobacteria-induced resistance by using the term SAR for the pathogen-induced type and ISR for the rhizobacteria-induced type of resistance. Figure 4.1 illustrates the broad spectrum effectiveness of both types of biologically induced resistance in Arabidopsis. Pathogen induced SAR requires SA, whereas rhizobacteria mediated ISR is almost always dependent on JA and ET signalling (Van Loon & Bakker, 2005). In the past decade, many components of the corresponding signalling cascades have been elucidated.

4.3.1 Systemic acquired resistance

The onset of SAR is associated with increased levels of SA both locally at the site of infection and systemically in distant tissues (Mauch-Mani & Métraux, 1998). Moreover, SAR is associated with the coordinate activation of a specific set of genes encoding pathogenesis related (PR) proteins, some of which possess antimicrobial activity (Van Loon, 1997). Exogenous application of SA, or its functional analogues 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole (BTH) induces SAR and activates the same set of *PR* genes (Ryals *et al.*, 1996; see also Chapter 2). Transgenic NahG plants that cannot accumulate SA, and the recessive mutants *sid1*, *sid2* and *pad4*, which are compromised in pathogen induced SA accumulation, are incapable of developing SAR and do not show

Figure 4.1 Spectrum of effectiveness of pathogen-induced SAR and rhizobacteria-mediated ISR in Arabidopsis. Photographs show typical symptoms caused by the respective pathogens and insects. SAR was induced by infiltrating three leaves per plant with avirulent *Pseudomonas syringae* pv. *tomato* DC3000(*avrRpt2*) bacteria, two days before challenge. ISR was induced by growing plants in soil containing ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria. The effectiveness of SAR and ISR against these pathogens is indicated with a red (SAR) or green (ISR) squares and was assessed on the basis of symptom severity (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997; Ton *et al.*, 2002c; V. van Oosten, M. Dicke, J.A. van Pelt and C.M.J. Pieterse, unpublished results).

PR gene activation upon pathogen infection (Gaffney *et al.*, 1993; Lawton *et al.*, 1995; Zhou *et al.*, 1998; Nawrath & Métraux, 1999). All together, this indicates that SA is a necessary intermediate in the SAR signalling pathway.

Many conditions have been described to induce SAR as well as defence related proteins (Van Loon, 2000). Particularly, the expression of a *PR-1* gene or protein is usually taken as a molecular marker to indicate that SAR was induced. All *PR-1* genes in plants appear to be inducible by SA, and endogenous production or exogenous application of SA has been shown to be both necessary and sufficient to elicit the induced state (Vernooij *et al.*, 1994). Pathogen induced synthesis of SA in tobacco is considered to occur from benzoate, whereas the evidence in Arabidopsis points to isochorismate as the immediate precursor (Wildermuth *et al.*, 2001). Although SA can be transported in the plant, reciprocal graftings of transgenic NahG plants, in which SA is degraded, and non-transformed plants as rootstocks or scions, demonstrated that SA is not the translocated signal in SAR (Vernooij *et al.*, 1994). Similar graftings between transgenic ET insensitive tobacco plants expressing a mutant ET receptor gene from Arabidopsis as rootstock and non-transformed control plants as scion showed little or no SAR induction in the scion, indicating that ET perception is necessary for the generation, release or transport of the mobile signal to distant tissues. Upon arrival of the mobile signal, the latter tissues must start producing SA, which induces the defence related proteins locally (Verberne *et al.*, 2003). The nature of the mobile signal has remained elusive so far. An Arabidopsis mutant, *dir1*, impaired specifically in the systemic character of SAR, implicates involvement of a lipid transfer protein (Maldonado *et al.*, 2002), suggesting that the mobile signal may contain a lipid moiety.

4.3.1.1 NPR1: a crucial regulatory protein of SAR

Transduction of the SA signal to activate *PR* gene expression and SAR requires the function of NPR1, also known as NIM1 (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). NPR1 is a regulatory protein identified in Arabidopsis through several genetic screens for SAR compromised mutants (Dong, 2004; Pieterse & Van Loon, 2004). During induction of SAR, NPR1 is translocated into the nucleus (Kinkema *et al.*, 2000). NPR1 acts as a modulator of *PR* gene expression but does not bind DNA directly (Després *et al.*, 2000). Yeast two-hybrid analyses indicated that NPR1 acts through members of the TGA subclass of the basic Leu zipper (bZIP) family of transcription factors (TGAs) that are implicated in the activation of target *PR* genes (Zhang *et al.*, 1999; Després *et al.*, 2000; Zhou *et al.*, 2000). Electromobility shift assays showed that NPR1 substantially increases binding of TGA2 to SA responsive promoter elements in the Arabidopsis *PR-1* gene (Després *et al.*, 2000), suggesting that NPR1-mediated DNA binding of TGAs is important for *PR* gene activation. Recently, microarray analyses showed that in addition to controlling the expression of *PR* genes, NPR1 also controls the expression of protein secretory pathway genes. Up-regulation of these genes is essential for SAR, because mutations in some of them diminished the secretion of PR proteins, with a concomitant reduction in the level of resistance (Wang *et al.*, 2005).

4.3.1.2 NPR1–TGA interactions in vivo

Evidence that binding between NPR1 and TGAs occurs *in planta* was provided in several studies. Subramaniam and co-workers used a protein fragment complementation assay to

demonstrate interactions between NPR1 and TGA2 *in vivo*, and showed that the SA induced interaction is predominantly localized in the nucleus (Subramaniam *et al.*, 2001). Fan & Dong (2002) followed a genetic approach, using Arabidopsis transgenics that over-expressed the C-terminal domain of TGA2. This mutant TGA2 protein was capable of interacting with NPR1, but lacked the DNA binding activity important for TGA function. Accumulation of the dominant-negative mutant TGA2 protein in a wild type background led to dose-dependent abolition of TGA function in an NPR1-dependent manner. The resulting phenotype resembled that of mutant *npr1* plants in that the ability to express *PR-1* in response to the SA-analogue 2,4-dichloroisonicotinic acid (INA) was impaired, and the susceptibility to infection by *Pseudomonas syringae* pv. *maculicola* was enhanced. Chromatin immunoprecipitation experiments revealed that *in vivo* both TGA2 and TGA3 are recruited in a SA- and NPR1-dependent manner to SA responsive elements in the *PR-1* promoter (Johnson *et al.*, 2003), supporting the notion that both these transcription factors can act as positive regulators of defence-related gene expression.

4.3.1.3 TGA function and redox regulation

Knockout analysis of single, double, and triple mutants of *TGA2*, *TGA5* and *TGA6* in various combinations established that these three TGAs play an essential and partially redundant role in the activation of *PR* gene expression and SAR in Arabidopsis (Zhang *et al.*, 2003). The seven known Arabidopsis TGAs show differential binding activity towards NPR1 in yeast two-hybrid assays, with TGA2, TGA3 and TGA6 showing the strongest binding (Zhang *et al.*, 1999; Després *et al.*, 2000; Zhou *et al.*, 2000). TGA1 and TGA4 did not bind to NPR1 in yeast assays. However, by using an *in planta* transient expression assay mechanistically similar to the yeast two-hybrid system, Després and co-workers demonstrated that TGA1 does interact with NPR1 in Arabidopsis leaves upon SA treatment (Després *et al.*, 2003). In the same study, yeast two-hybrid assays with chimeric TGA1 proteins in which various domains were exchanged with TGA2 revealed that a 30 amino acid segment is important for NPR1 interaction. Amino acid sequence comparison with other TGAs revealed that both TGA1 and TGA4 contain two Cys residues in this region that are missing in the TGAs that interact with NPR1 in yeast. Mutation of these Cys residues to Asn and Ser transformed TGA1 and TGA4 into proteins capable of interacting with NPR1 in yeast. Because the Cys residues can form disulfide bridges that might prevent interaction of TGA1 and TGA4 with NPR1, Després *et al.* (2003) tested whether the *in vivo* redox state of TGA1 affects NPR1 binding. Upon treatment of Arabidopsis leaves with SA, the Cys residues of TGA1 were reduced, thereby facilitating interaction with NPR1 and subsequent enhancement of binding of TGA1 to SA responsive promoter elements.

4.3.1.4 Redox changes: connection between the SA signal and NPR1 functioning

NPR1 plays an important role in the SA mediated activation of defence related genes by enhancing DNA binding of TGAs to SA responsive elements in their promoters. But how does NPR1 transduce the SA signal? Previously, experiments with NPR1/NIM1 over-expressers

demonstrated that high levels of NPR1 proteins per se do not induce *PR*-gene expression or resistance, indicating that NPR1 needs to be activated by a factor acting downstream of SA (Cao *et al.*, 1998; Friedrich *et al.*, 2001). Observations that NPR1 proteins from different plant species contain conserved Cys residues capable of forming inter- or intra-molecular disulfide bonds, and that a mutation in one of these Cys residues resulted in a mutant *npr1* phenotype, led Mou *et al.* (2003) to the hypothesis that NPR1 protein conformation might be sensitive to SA-induced changes in cellular redox status. Induction of SAR was indeed shown to be associated with a change in redox state, possibly caused by accumulation of antioxidants. Under these conditions, NPR1 was reduced from an inactive oligomeric complex to an active monomeric form. The latter appeared to be required for *PR-1* gene activation, as inhibition of NPR1 reduction prevented *PR-1* gene expression. Mutation of the two Cys residues critical for NPR1 oligomer formation led to constitutive monomerization and nuclear localization of NPR1, as well as constitutive *PR-1* gene expression. Thus, cellular redox changes induced as a result of SA action connect the SA signal with NPR1 activity during SAR. Figure 4.2 summarizes the important steps in SAR signalling.

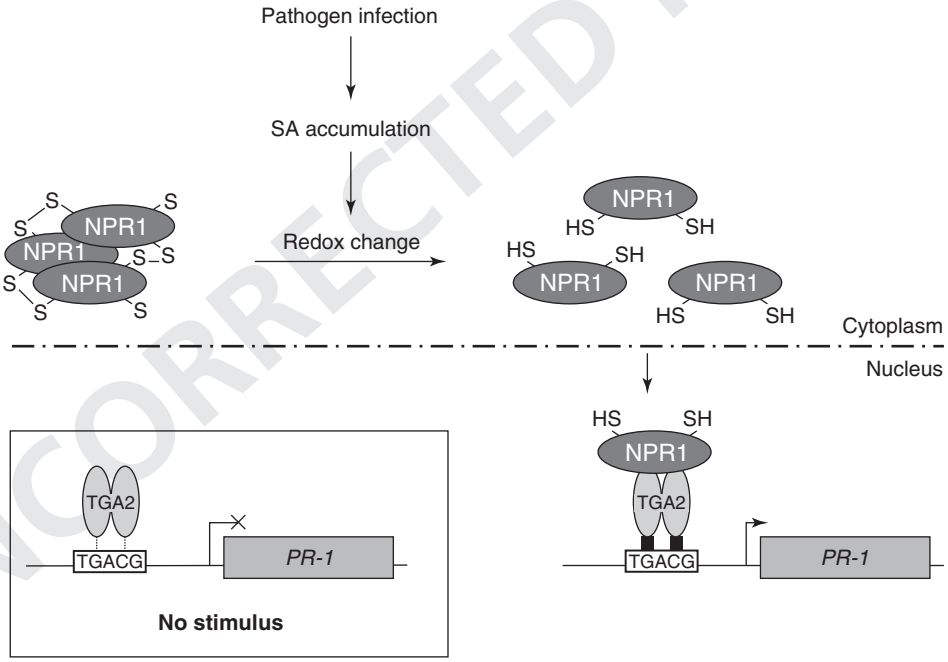

Figure 4.2 Model for SAR signalling illustrating the role of SA-mediated redox changes, NPR1, and TGA transcription factors in SAR-related gene expression. In non-induced cells, oxidized NPR1 is present as inactive oligomers that remain in the cytosol. Binding of TGAs to the cognate SA-responsive promoter elements (TGACG) does not activate *PR-1* gene expression (insert). Upon infection by a necrotizing pathogen, SA accumulates and plant cells attain a more reducing environment, possibly due to the accumulation of antioxidants. Under these conditions, NPR1 oligomers are reduced to an active monomeric state through reduction of intermolecular disulfide bonds. Monomeric NPR1 is translocated into the nucleus where it interacts with TGAs, such as TGA2. The binding of NPR1 to TGAs increases the DNA-binding activity of these transcription factors to the cognate *cis* element (black boxes), resulting in the activation of *PR-1* gene expression (adapted from Pieterse & Van Loon, 2004).

4.3.2 Rhizobacteria-induced systemic resistance

Plants produce exudates and lysates at their root surface, where rhizobacteria are attracted in large numbers (Lynch & Whipps, 1991; Lugtenberg *et al.*, 2001; Walker *et al.*, 2003). Selected strains of non-pathogenic rhizobacteria appear to be plant growth promoting, because they possess the capability to stimulate plant growth (Kloepper *et al.*, 1980; Pieterse & Van Loon, 1999; Bloemberg & Lugtenberg, 2001). Although direct effects on plant growth have been reported (Lynch, 1976; Van Peer & Schippers, 1989), growth promotion results mainly from the suppression of soil borne pathogens and other deleterious micro-organisms (Schippers *et al.*, 1987). Fluorescent *Pseudomonas* spp. are among the most effective plant growth promoting rhizobacteria and have been shown to be responsible for the reduction of soil borne diseases in naturally disease suppressive soils (Raaijmakers & Weller, 1998; Weller *et al.*, 2002; Duff *et al.*, 2003). This type of natural biological control can result from competition for nutrients, siderophore mediated competition for iron, antibiosis or the production of lytic enzymes (Bakker *et al.*, 1991; Van Loon & Bakker, 2003). Apart from such direct antagonistic effects on soil borne pathogens, some rhizobacterial strains are also capable of reducing disease incidence in above ground plant parts through a plant mediated defence mechanism called ISR (Van Loon *et al.*, 1998; Chapter 8). Like SAR, rhizobacteria mediated ISR has been demonstrated in many plant species, e.g. bean, carnation, cucumber, radish, tobacco, tomato and the model plant *Arabidopsis thaliana*, and is effective against a broad spectrum of plant pathogens, including fungi, bacteria and viruses (Van Loon *et al.*, 1998).

Several bacterially derived compounds have been implicated in the elicitation of ISR (Van Loon *et al.*, 1998; Bakker *et al.*, 2003; Van Loon & Bakker, 2005). Elicitors comprise cell wall components such as lipopolysaccharides and flagella, as well as metabolites, such as siderophores and antibiotics (Van Peer & Schippers, 1992; Leeman *et al.*, 1995b; Van Wees *et al.*, 1997; Bakker *et al.*, 2003; Iavicoli *et al.*, 2003). Whereas a receptor for bacterial flagellin has been identified (Gomez-Gomez & Boller, 2000), putative receptors for bacterial cell wall preparations have not been isolated. However, the striking homologies with sensitive perception mechanisms for pathogen associated molecular patterns (PAMPS) that function in the innate immune response of plants and animals (Nürnberger *et al.*, 2004) suggest that rhizobacteria are recognized by general immune surveillance mechanisms.

4.3.2.1 ISR in Arabidopsis: discovery of an SA independent signalling cascade

To study the signal transduction pathway of rhizobacteria mediated ISR, an Arabidopsis based model system was developed. In this model system, the non-pathogenic rhizobacterial strain *Pseudomonas fluorescens* WCS417r is used as the inducing agent (Pieterse *et al.*, 1996). WCS417r has been shown to trigger ISR in several plant species, e.g. carnation, radish, tomato and bean (Pieterse *et al.*, 2002). Colonization of Arabidopsis roots by ISR-inducing WCS417r bacteria protects the plants against different types of pathogens, including the bacterial leaf pathogens *Pst* DC3000, *X. campestris* pv. *armoraciae*, and *E. carotovora* pv. *carotovora*, the fungal root pathogen *Fusarium oxysporum* f. sp. *raphani*, the fungal leaf pathogens *A. brassicicola* and *B. cinerea,* and the

oomycete leaf pathogen *H. parasitica* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997; Ton *et al.*, 2002a; H.J.A.Van Pelt & C.M.J. Pieterse, unpublished results).

Research on the molecular mechanism of rhizobacteria-mediated ISR was initially focused on the role of PR-proteins, as the accumulation of these proteins was considered to be strictly correlated with induced disease resistance. However, radish plants of which the roots were treated with ISR-inducing WCS417r did not accumulate PR proteins, although these plants clearly showed enhanced resistance against fusarium wilt disease (Hoffland *et al.*, 1995). Similarly, Arabidopsis plants expressing WCS417r-mediated ISR showed enhanced resistance against *F. oxysporum* f. sp. *raphani* and *Pst* DC3000, but this did not coincide with the activation of the SAR marker genes *PR-1*, *PR-2* and *PR-5* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Determination of SA levels in ISR-expressing Arabidopsis plants revealed that ISR is not associated with increased accumulation of SA (Pieterse *et al.*, 2000). Moreover, WCS417r-mediated ISR was expressed normally in SA-non-accumulating Arabidopsis NahG plants (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). This led to the conclusion that WCS417r-mediated ISR is an SA-independent resistance mechanism and that WCS417r-mediated ISR and pathogen induced SAR are regulated by distinct signalling pathways. SA independent ISR has been shown not only in Arabidopsis (Van Wees *et al.*, 1997; Iavicoli *et al.*, 2003; Ryu *et al.*, 2003) but also in tobacco (Press *et al.*, 1997; Zhang *et al.*, 2002), and tomato (Yan *et al.*, 2002). This wide range of induction of ISR indicates that the ability of these *Pseudomonas* strains to activate an SA-independent pathway controlling systemic resistance is common to a broad range of plants.

Not all ISR-inducing rhizobacteria trigger an enhanced defensive capacity via an SA-independent pathway. Under iron limiting conditions, certain rhizobacterial strains produce SA as a siderophore (Meyer *et al.*, 1992; Visca *et al.*, 1993). An enhanced resistance elicited by *P. fluorescens* CHA0 in tobacco might be fully explained by the bacterial production of SA, which could elicit a SAR response. Treatment of tobacco roots with CHA0 triggers accumulation of SA-inducible PR-proteins in the leaves (Maurhofer *et al.*, 1994). Moreover, transformation of the SA-biosynthetic gene cluster of CHA0 into *P. fluorescens* P3 improved the systemic resistance-inducing capacity of this strain (Maurhofer *et al.*, 1998). Another strain that elicits an SA-dependent enhanced defensive capacity is *Pseudomonas aeruginosa* 7NSK2. An SA-deficient mutant of this bacterium failed to induce resistance in bean and tobacco (De Meyer & Höfte, 1997). Moreover, 7NSK2 was unable to induce resistance in NahG tobacco plants against tobacco mosaic virus (De Meyer *et al.*, 1999). An SA-overproducing mutant of 7NSK2 was shown to trigger the SA-dependent SAR pathway by producing SA at the root surface (De Meyer & Höfte, 1997). However, Audenaert *et al.* (2002b) showed that a combination of the secondary siderophore pyochelin and the antibiotic pyocyanin is required to induce enhanced resistance by wild-type 7NSK2. SA is an intermediate in the formation of pyochelin, and the combination of pyochelin and pyocyanin is toxic to root cells, thereby setting off the SAR response.

4.3.2.2 Genetic dissection of the SA-independent ISR signalling cascade

ISR-inducing rhizobacteria show little specificity in their colonization of roots of different plant species (Van Loon *et al.*, 1998). In contrast, the ability to induce ISR appears to be dependent on the bacterium/host combination. For instance, *P. fluorescens* WCS374r is capable of inducing ISR in radish, but not in Arabidopsis (Leeman *et al.*, 1995a;

Van Wees *et al.*, 1997). Conversely, Arabidopsis is responsive to *Pseudomonas putida* WCS358r, whereas radish is not (Van Peer *et al.*, 1991; Van Peer & Schippers, 1992; Leeman *et al.*, 1995a; Van Wees *et al.*, 1997). WCS417r is capable of inducing ISR in both Arabidopsis and radish (Van Wees *et al.*, 1997), as well as in other species, i.e. carnation (Van Peer *et al.*, 1991), radish (Leeman *et al.*, 1995a), tomato (Duijff *et al.*, 1998) and bean (Bigirimana & Höfte, 2002), but not in *Eucalyptus* (Ran *et al.*, 2005). Besides differences in inducibility between species, there can also be differences within species. Arabidopsis accessions Columbia (Col-0) and Landsberg *erecta* (L*er*-0) are responsive to ISR induction by WCS417r, but accessions Wassilewskija (Ws-0) and RLD1 are not (Van Wees *et al.*, 1997; Ton *et al.*, 1999, 2001). Both these accessions are compromised in a common trait governing a step between the recognition of the bacterium and the expression of ISR. These data clearly indicate that ISR is genetically determined.

Since SA was not involved in WCS417r-elicited ISR, the Arabidopsis JA-response mutant *jar1* and the ET-response mutant *etr1* were tested for their ability to express ISR. Both mutants were unable to mount resistance against *Pst* DC3000 after colonization of the roots by WCS417r (Pieterse *et al.*, 1998), indicating that ISR requires responsiveness to both JA and ET. Another indication for the involvement of the JA-signalling pathway came from the analysis of Arabidopsis mutant *eds8*, which was previously shown to exhibit enhanced susceptibility to *P. syringae* (Glazebrook *et al.*, 1996). This mutant was impaired in both WCS417r-mediated ISR (Ton *et al.*, 2002d) and JA-signalling (Ton *et al.*, 2002c; Glazebrook *et al.*, 2003). To further elucidate the role of ET in the ISR signalling pathway, a large set of well characterized ET-signalling mutants was analysed. None of these mutants showed an ISR response against *Pst* DC3000 after colonization of the roots by WCS417r (Knoester *et al.*, 1999). These results confirmed that an intact ET-signalling pathway is required for the establishment of ISR. Particularly interesting was the analysis of the *eir1* mutant, which is ET-insensitive in the roots, but not in the shoot. This *eir1* mutant was incapable of showing ISR after root colonization by WCS417r. In contrast, after leaf infiltration with WCS417r, it did show ISR, indicating that responsiveness to ET is required at the site of rhizobacterial induction (Knoester *et al.*, 1999).

Further evidence for the involvement of the ET-response pathway came from the identification of the Arabidopsis *ISR1* locus (Ton *et al.*, 1999). Genetic analysis of the progeny of a cross between the WCS417r-responsive ecotype Col-0 and the ISR-impaired ecotype RLD1 revealed a single locus, designated *ISR1*, to be important in the expression of ISR against several different pathogens (Ton *et al.*, 2002b). Accessions with the recessive *isr1* allele have reduced sensitivity to ET and enhanced susceptibility to *Pst* DC3000 (Ton *et al.*, 2001). These results strongly indicate that the Arabidopsis *ISR1* locus encodes a novel component in the ET-signal transduction pathway that is important for both basal resistance and ISR in Arabidopsis.

4.3.2.3 Dual role for NPR1 in SAR and ISR

To investigate a possible involvement of the SAR regulatory protein NPR1 in ISR signalling, the *Arabidopsis npr1* mutant was tested in the ISR bioassay. Surprisingly, the *npr1* mutant was incapable of showing WCS417r-mediated ISR (Pieterse *et al.*, 1998; Van Wees *et al.*, 2000). This result clearly showed that WCS417r-mediated ISR, like SA-dependent SAR, is an NPR1-dependent defence response. Further analysis of the ISR signal-transduction

pathway revealed that NPR1 acts downstream of the JA- and ET-dependent steps (Pieterse *et al.*, 1998). Because SAR is associated with NPR1-dependent *PR*-gene expression, and ISR is not, the action of NPR1 in ISR must be different from that in SAR. These different activities are not mutually exclusive because simultaneous activation of ISR and SAR can lead to an enhanced defensive activity compared to that observed with either type of induced resistance alone (Van Wees *et al.*, 2000). These results suggest that the NPR1 protein is important in regulating and intertwining different hormone-dependent defence pathways.

4.3.2.4 ISR is associated with priming for enhanced defence

In Arabidopsis, both JA and ET activate specific sets of defence-related genes (Schenk *et al.*, 2000), but when applied exogenously, each can induce resistance (Pieterse *et al.*, 1998; Van Wees *et al.*, 1999). To investigate how far ISR is associated with these changes in JA/ET-responsive gene expression, Van Wees *et al.* (1999) monitored the expression of a set of well characterized JA- and/or ET-responsive, defence-related genes (i.e. *LOX1*, *LOX2*, *VSP2*, *PDF1.2*, *HEL*, *CHI-B* and *PAL1*) in Arabidopsis plants expressing WCS417r-mediated ISR. None of these genes was up-regulated in induced plants, neither locally in the roots nor systemically in the leaves. This suggested that the resistance attained was not associated with major increases in the levels of either JA or ET. Indeed, analysis of JA and ET levels in leaves of ISR-expressing plants revealed no changes in the production of these signal molecules (Pieterse *et al.*, 2000; Hase *et al.*, 2003). Therefore, it had to be assumed that the JA and ET dependency of ISR is based on an enhanced sensitivity to these hormones, rather than on an increase in their production.

To identify ISR-related genes, the transcriptional response of over 8000 Arabidopsis genes was monitored during WCS417r-mediated ISR (Verhagen *et al.*, 2004). However, systemically in the leaves, none of the ~8000 genes tested showed a consistent change in expression in response to effective colonization of the roots by WCS417r, indicating that the onset of ISR in the leaves is not associated with detectable changes in gene expression. However, after challenge inoculation of WCS417r-induced plants with the bacterial leaf pathogen *Pst* DC3000, 81 genes showed an augmented expression pattern in ISR-expressing leaves compared to inoculated control leaves, suggesting that ISR-expressing plants are primed to respond faster and/or more strongly upon pathogen attack. The majority of the primed genes was predicted to be regulated by JA and/or ET signalling, confirming earlier findings that colonization of the roots by WCS417r primed Arabidopsis plants for augmented expression of the JA- and/or ET-responsive genes *AtVSP2, PDF1.2* and *HEL* (Van Wees *et al.*, 1999; Hase *et al.*, 2003). Priming is a phenomenon that has been shown to be associated with different types of induced resistance (Conrath *et al.*, 2002). It provides the plant with an enhanced capacity for rapid and effective activation of cellular defence responses once a pathogen is contacted, and it allows the plant to react more effectively to any invader encountered by boosting the defences that are activated in the host. This mechanism could also explain the broad-spectrum action of induced resistance.

The first evidence that priming for potentiated expression of plant defence responses plays an important role in rhizobacteria-mediated ISR came from experiments with carnation. Upon colonization of the roots by WCS417, carnation plants developed an enhanced defensive capacity against *Fusarium oxysporum* f. sp. *dianthi*. Before challenge inoculation, no increase in phytoalexin levels could be detected in induced plants, but

upon subsequent inoculation with *F. oxysporum*, phytoalexin levels in ISR-expressing plants rose significantly faster than upon challenge of non-induced plants (Van Peer *et al.*, 1991). In bean, *Bacillus pumilus* SE34 induced ISR against the root-rot fungus *F. oxysporum* f. sp. *pisi* (Benhamou *et al.*, 1996). By itself, colonization of the roots by the rhizobacterium did not induce morphological alterations of root tissue. However, upon challenge with *F. oxysporum*, root cell walls of ISR-expressing plants were rapidly strengthened at sites of attempted fungal penetration by appositions containing large amounts of callose and phenolic materials, thereby effectively preventing fungal ingress (Benhamou *et al.*, 1996). Other ISR-inducing rhizobacteria have also been demonstrated to enhance the plant's defensive capacity by priming for potentiated defence-related gene expression (e.g. De Meyer *et al.*, 1999; Ahn *et al.*, 2002; Kim *et al.*, 2004; Tjamos *et al.*, 2005), indicating that priming is a common feature in rhizobacteria-mediated ISR. Priming for defence may combine advantages of enhanced disease protection with low metabolic costs. Recently,

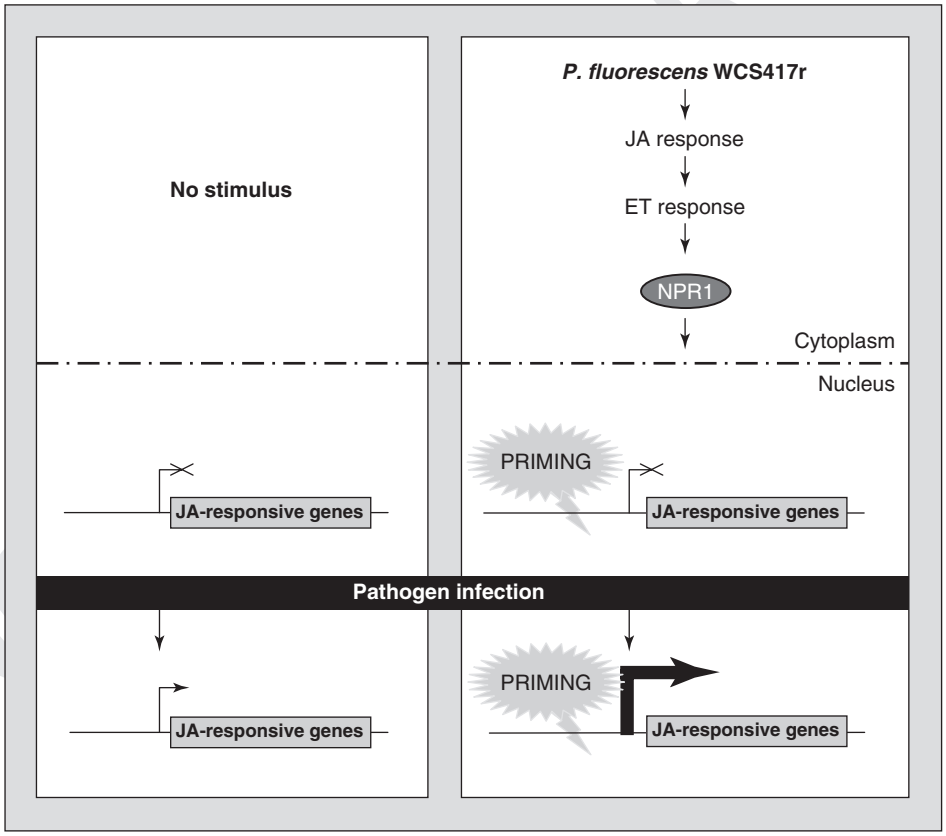

Figure 4.3 Model for the signal-transduction pathway controlling rhizobacteria-mediated ISR in Arabidopsis. Colonization of the roots by *P. fluorescens* WCS417r leads to enhanced defensive capacity against a broad spectrum of plant pathogens. For the expression of ISR, responsiveness to the plant hormones JA and ET are required, as well as the regulatory protein NPR1. The induced state is not associated with major changes in defence-related gene expression (as opposed to SAR). However, ISR-expressing plants are primed to express a specific set of JA-responsive genes faster and to a higher level upon pathogen infection.

Van Hulten *et al.* (2006) examined the costs and benefits of priming in comparison to activated defence in Arabidopsis. The study revealed that the benefits of priming-mediated resistance outweigh the costs under conditions of pathogen pressure, suggesting an evolutionary advantage of this mechanism of induced resistance over constitutive activation of defence responses. Figure 4.3 summarizes the key steps in the ISR signalling pathway.

4.4 Crosstalk between signalling pathways

In the induction of systemic resistance in Arabidopsis against *Pst* DC3000, SA-inducible SAR and JA/ET-dependent ISR can act additively. However, both pathways can also interact antagonistically, indicating that signalling pathways cross-communicate (Reymond & Farmer, 1998; Pieterse & Van Loon, 1999; Felton & Korth, 2000; Feys & Parker, 2000; Dicke & Van Poecke, 2002; Kunkel & Brooks, 2002; Rojo *et al.*, 2003; Bostock, 2005). For instance, activation of SA-dependent SAR has been shown to suppress JA signalling in plants, thereby prioritizing SA-dependent resistance to microbial pathogens over JA-dependent defence that is, in general, more effective against insect herbivory (Stout *et al.*, 1999; Thaler *et al.*, 1999; Felton & Korth, 2000; Thaler *et al.*, 2002; Bostock, 2005). Pharmacological and genetic experiments have indicated that SA-mediated suppression of JA-inducible gene expression plays an important role in this process (Peña-Cortés *et al.*, 1993; Van Wees *et al.*, 1999; Glazebrook *et al.*, 2003). Crosstalk can sometimes work in both directions, as evidenced by occasional suppression of SA responses by JA (Niki *et al.*, 1998; Kunkel & Brooks, 2002; Glazebrook *et al.*, 2003).

4.4.1 Complexity of the plant's induced resistance signalling network

To understand how plants integrate pathogen- and insect-induced signals into specific defence responses, De Vos *et al.* (2005) monitored the dynamics of SA, JA and ET signalling in *Arabidopsis* after attack by a set of microbial pathogens and herbivorous insects with different modes of attack. *Arabidopsis* plants were exposed to microbial pathogens (*Pst* DC3000 and *A. brassicicola*), tissue chewing caterpillars (*Pieris rapae*), cell content feeding thrips (*Frankliniella occidentalis*), or phloem feeding aphids (*Myzus persicae*). Monitoring the 'signal signature' in each plant – attacker combination showed that the kinetics of SA, JA and ET production vary greatly in both quantity and timing. Analysis of global gene expression profiles demonstrated that the signal signature characteristic of each *Arabidopsis* – attacker combination is orchestrated into a surprisingly complex set of transcriptional alterations in which, in all cases, stress related genes are over-represented. Comparison of transcript profiles revealed that consistent changes induced by pathogens and insects with very different modes of attack can show considerable overlap. Of all consistent changes induced by *A. brassicicola*, *P. rapae* and *F. occidentalis*, more than 50% were also induced consistently by *Pst* DC3000. However, although these four attackers all stimulated JA biosynthesis, the majority of the changes in JA-responsive gene expression were attacker specific. Hence, SA, JA and ET play a primary role in the orchestration of the plant's defence response, but other regulatory mechanisms, such as pathway crosstalk or additional attacker-induced signals, eventually shape the highly complex attacker-specific defence response.

4.4.2 Trade-offs between different types of induced resistance

Several studies have shown that activation of a particular defence pathway by one particular pathogen or insect negatively affects resistance to other groups of pathogens or insects. For instance, Moran (1998) demonstrated that in cucumber, pathogen-induced SAR against the fungus *Colletotrichum orbiculare* was associated with reduced resistance against feeding by the spotted cucumber beetle *Diabrotica undecimpunctata howardi* and enhanced reproduction of the melon aphid *Aphis gossypii*. A similar phenomenon was observed by Preston *et al.* (1999), who demonstrated that TMV-inoculated tobacco plants expressing SAR were more suitable for grazing by the tobacco hornworm *Manduca sexta* than non-induced control plants. Conversely, Felton *et al.* (1999) demonstrated that transgenic tobacco plants with reduced SA levels as a result of silencing of the *PAL* gene exhibited reduced SAR against TMV but enhanced herbivore-induced resistance to *Heliothis virescens* larvae. In contrast, *PAL*-overexpressing tobacco plants showed a strong reduction in herbivore-induced insect resistance, while TMV-induced SAR was enhanced in these plants.

Application of the SAR inducer acibenzolar-*S*-methyl (BTH) has been shown to negatively affect insect resistance as well. For instance, BTH induced resistance against the bacterial pathogen *P. syringae* pv. *tomato*, but improved suitability of tomato leaves for feeding by leaf chewing larvae of the corn earworm *Helicoverpa zea* (Stout *et al.*, 1999). A similar phenomenon was observed by Thaler *et al.* (1999), who showed that application of BTH to field-grown tomato plants compromised resistance to the beet armyworm (*Spodoptera exigua*). In most cases, reduced insect resistance observed in SAR-expressing plants is attributed to the inhibition of JA production by BTH or increased SA levels.

4.4.3 Concomitant expression of induced defence pathways

Whereas negative interactions between pathogen and insect resistance have been clearly demonstrated, other studies failed to demonstrate such a negative relationship. For instance, Ajlan & Potter (1992) found that inoculation of the lower leaves of tobacco with TMV had no effect on population growth of tobacco aphids (*Myzus nicotianae*). Similarly, Inbar *et al.* (1998) found no negative effect of BTH application on population growth of whiteflies (*Bemisia argentifolii*) and leaf miners (*Liriomyza* spp.). However, Stout *et al.* (1999) showed that inoculation of tomato leaves with the bacterial pathogen *P. syringae* pv. *tomato* induced resistance against both *P. syringae* pv. *tomato* and the corn earworm in distal plant parts. Conversely, feeding by the insect *H. zea* likewise induced resistance against both *P. syringae* pv. *tomato* and itself.

A demonstration of induced resistance effective simultaneously against pathogens and insects in the field was provided by Zehnder *et al.* (2001). In cucumber, induction of rhizobacteria-mediated ISR against the insect-transmitted bacterial wilt disease, caused by *Erwinia tracheiphila*, was associated with reduced feeding of the cucumber beetle vector. It appeared that induction of ISR was associated with reduced concentrations of cucurbitacin, a secondary plant metabolite and powerful feeding stimulant for cucumber beetles. Induction of ISR against *E. tracheiphila* was also effective in the absence of beetle vectors, suggesting that ISR protects cucumber against bacterial wilt not only by reducing beetle feeding and transmission of the pathogen, but also through the induction of defence responses that are active against the pathogen itself. These observations indicate that negative interactions between induced pathogen and insect resistance are by no means general.

4.4.4 Key players in pathway crosstalk

The antagonistic effect of SA on JA signalling was recently shown to be controlled by a novel function of the defence regulatory protein NPR1 (Spoel *et al.*, 2003). The nuclear localization of NPR1 that is essential for SA-mediated *PR*-gene expression appeared not to be required for the suppression of JA signalling. Thus, crosstalk between SA and JA is modulated through a novel function of NPR1 in the cytosol (Spoel *et al.*, 2003). The mode of action of NPR1 in the cytosol is unknown, but it is tempting to speculate that it interferes with the previously identified SCF^{COI1} ubiquitin-ligase complex (Devoto *et al.*, 2002; Xu *et al.*, 2002) that regulates JA responsive gene expression through targeted ubiquitination and subsequent proteasome-mediated degradation of a negative regulator of JA signalling.

Additional key elements involved in pathway crosstalk have been identified. For instance, 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 antagonistic pathways (Li *et al.*, 2004). In addition, the transcription factors ERF1 and MYC2 were found to integrate signals from the JA and ET pathway in activating defence-related genes that are responsive to both JA and ET (Lorenzo *et al.*, 2003, 2004). Crosstalk between defence signalling pathways is thought to provide the plant with a powerful regulatory potential, which helps the plant to 'decide' on the most appropriate defensive strategy, depending on the type of attacker it is encountering. Yet, it may also allow attackers to manipulate plants to their own benefit by shutting down induced defence through influences on the signalling network (Kahl *et al.*, 2000).

4.5 Outlook

Plant diseases are responsible for large crop losses in agriculture. Conventional disease control is based on resistance breeding and application of chemical agents. Classic resistance breeding depends on the availability of resistance genes, which often show limited durability. The use of chemical agents and their persistence in soil are potentially harmful to the environment, notably when chemicals are applied repeatedly in large amounts such as in the control of soil-borne fungal pathogens. Moreover, both these disease control strategies are directed against a single or a small group of plant pathogens. Induced disease resistance is an attractive alternative form of plant protection, as it is based on the activation of extant resistance mechanisms in the plant and is effective against a broad spectrum of plant pathogens (Kuć, 1982; Van Loon *et al.*, 1998).

Previously, Van Wees *et al.* (2000) demonstrated that simultaneous activation of ISR and SAR results in an enhanced level of induced protection against *Pst* DC3000. It appeared that the JA/ET-dependent ISR pathway and the SA-dependent SAR pathway act independently and additively to increase protection against this particular pathogen. Moreover, ISR and SAR confer differential protection against biotrophic and necrotrophic pathogens (Ton *et al.*, 2002c). Thus, combining both types of induced resistance can protect the plant against a complementary spectrum of pathogens and can result in an additive level of induced protection against pathogens that are resisted through both the JA/ET- and the SA-dependent pathways, such as *Pst* DC3000. Hence, combining SAR and ISR provides an attractive tool for improvement of disease control.

Knowledge of defence signalling pathways has been proven to be instrumental for the development of new strategies for broad-spectrum disease resistance. Examples are genetic

engineering of the SAR pathway, and the development of defence signal-mimicking chemicals, such as BTH. However, crosstalk between SA- and JA-dependent defence pathways may be a burden when enhanced pathogen resistance is associated with reduced resistance against insects. Fortunately, negative crosstalk between SA- and JA-dependent defences appears to be confined to specific inducer – plant – attacker combinations. Only in cases in which the inducer strongly activates the SAR pathway does there seem to be an antagonistic effect on resistance against attackers that are resisted through JA-dependent defences. In other cases, there seems to be little or no antagonism, and SA- and JA-dependent defences can be expressed concomitantly to boost the plant's potential to resist invaders. Thus, the general notion that SA-dependent pathogen resistance and JA-dependent insect resistance are mutually exclusive needs to be adjusted.

Future research on the molecular mechanisms of induced resistance and crosstalk between plant defence pathways will provide more insight into how plants are able to integrate signals into appropriate defences. Ultimately, this will not only provide fundamental insights into how plants cope with different enemies, but also be instrumental in developing strategies for biologically based, environmentally friendly and durable crop protection.

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