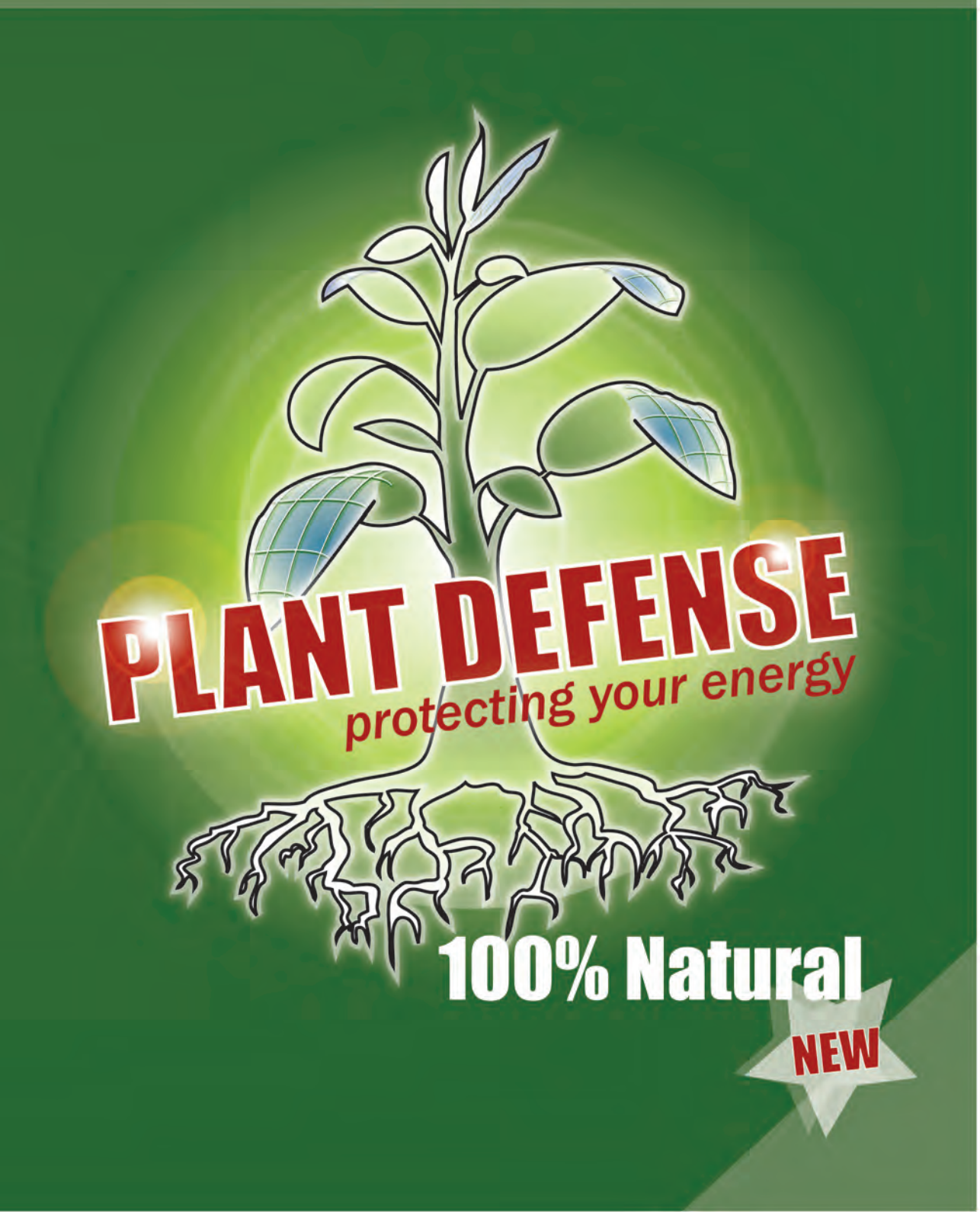
Transcriptional regulators of rhizobacteria-induced systemic resistance

Sjoerd van der Ent



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Transcriptional regulators of rhizobacteria-induced systemic resistance

Transcriptionele regulatoren van door rhizobacteriën geïnduceerde systemische resistentie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
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des middags te 12.45 uur
door

Sjoerd van der Ent
geboren op 28 december 1979 te Rotterdam

Promotoren: Prof.dr.ir. C.M.J. Pieterse
Prof.dr.ir. L.C. Van Loon

Co-promotor: Dr. J. Ton

For Maarteke

If I have the gift of prophecy, and know all mysteries and all knowledge;
and if I have all faith, so as to remove mountains,
but do not have love,
I am nothing.
[1 Cor. 13:2]

CONTENTS

CHAPTER 1

General Introduction

Adapted from:

Van der Ent, S., Koornneef, A., Ton, J. and Pieterse, C.M.J. (2008). Induced resistance - orchestrating defence mechanisms through cross-talk and priming. *In*: Annual Plant Reviews - Molecular Aspects of Plant Disease Resistance (J.E. Parker, ed.), Blackwell, *in press*

Plants are of crucial importance for life on Earth. Through the process of photosynthesis, plants are capable of converting inorganic molecules into organic compounds by using solar energy. These organic compounds provide the energy required for all active processes not only within the plant itself, but also for all organisms at higher trophic levels. Furthermore, photosynthesis leads to the emission of oxygen, which is indispensable for all respiring organisms. Moreover, plant life has an enormous influence on weather and climate, as three-quarters of vaporized water from global land surface results from plant transpiration (Von Caemmerer & Baker, 2007). Hence, plants shape the biosphere of our planet.

PLANTS IN THEIR ENVIRONMENT

During their life cycle, plants tightly regulate many vital processes, such as photosynthesis, respiration, water balance, uptake of nutrients, and transport of nutrients and photo-assimilates. Continuation of each of these is required for survival, yet some of these processes affect each other negatively. For example, gas exchange and maintenance of water balance are essential for photosynthesis, transport and growth. However, the uptake of CO_2 through stomata for photosynthesis inevitably leads to water loss through transpiration. To allow proper continuation of both processes, they are tightly integrated through interconnected signaling pathways that are regulated by multiple endogenous and environmental cues, such as the phytohormone abscisic acid (ABA) and light, respectively. Nevertheless, the resulting trade-offs limit plant growth and development even under the most favorable environmental conditions. Moreover, plants rarely live under optimal conditions. Most of the time, they must cope with a variety of environmental stresses, such as extremes in temperature, humidity, salinity, pH or nutrient availability. In the course of evolution, plants have evolved mechanisms to survive under such stressful environmental conditions. Desert climates have resulted in the occurrence of water-saving CAM (crassulacean acid metabolism) plants, while carnivorous plants have evolved to trap e.g. insects as a source of nutrients in habitats where nutrients are limiting. Furthermore, all plant species are equipped with tightly coordinated signaling cascades that allow them to respond adequately to the continuously fluctuating abiotic stresses in their environment. The complexity of these regulatory pathways provides the plant with a surprising flexibility, to the extent that it has been interpreted as a form of plant intelligence (Trewavas, 2003).

BIOTIC STRESS

Apart from abiotic stresses, plants have to deal with many sources of biotic stress. In the first place, they have to compete with other plants for the same pool of nutrients and light. Plants are obliged to allocate assimilation products to either root or shoot tissue, to

improve competitiveness for nutrients and light, respectively, to ensure survival and reproductive success. Timely perception of putative competitors is of crucial importance in order to successfully compete with neighboring plants (Vandenbussche *et al.*, 2005; Pierik *et al.*, 2007). Secondly they face parasitism and predation. Parasitic plants, such as witchweed (*Striga* spp.) and broomrape (*Orobanche* spp.), are specialized in deriving nutrients and organic compounds from non-parasitic plants (Press & Gurney, 2000). In addition, many heterotrophic organisms are specialized in exploiting plants as a source of energy. Grazing vertebrates and tissue-chewing, cell-content feeding, or phloem-sucking insects are well-known examples at the macroscopic level (Schoonhoven *et al.*, 2005). On a microscopic scale, even more threats are present in the form of fungi, bacteria, oomycetes and nematodes (Agrios, 2005). All these organisms aim to exploit the plant's photosynthetic products, either by parasitizing living plants (biotrophs), or by killing plants and using the dead tissues as a food source (necrotrophs).

PLANT RESISTANCE: ORCHESTRATING DEFENSE MECHANISMS

BASAL RESISTANCE: CONSTITUTIVE AND INDUCIBLE DEFENSES

The common observation that plants are resistant to the majority of potentially harmful micro-organisms and arthropods suggests an extensive array of defensive mechanisms. Some of these mechanisms are pre-existing, and prevent or attenuate invasion by potential attackers. Thorns, needles and trichomes are examples of constitutive defensive structures that are designed to harm or deter herbivores. On a smaller scale, the cell wall poses a physical barrier to many micro-organisms. Many plants constitutively produce secondary metabolites that render the tissue less attractive to micro-organisms and herbivores (Osbourn, 1996; Tierens *et al.*, 2001). Despite the diversity of pre-existing defensive barriers, many micro-organisms and insects are still able to overcome this layer of defense. In such a situation, a wide spectrum of inducible plant defenses becomes activated, which aim to prevent the attacker from causing further damage, either by physically blocking tissue colonization, or by directly targeting the attacker's physiology. Because these inducible defenses are metabolically costly (Heil, 2002; Walters & Boyle, 2005; Van Hulten *et al.*, 2006), plants have evolved sophisticated regulatory mechanisms to orchestrate their inducible defense in a cost-efficient manner.

Induced defense against insects

Induced defense against insects can be divided roughly into two categories. The first involves a systemically increased production of proteinase inhibitors, toxins or feeding deterrents that target the attacking insect directly (Ryan, 1992; Kessler & Baldwin, 2002; Howe, 2004). Remarkably, some of these direct defenses can even be triggered by egg

deposition through the recognition of insect-derived bruchins (Doss *et al.*, 2000; Little *et al.*, 2007). The second category of induced defense against insects aims to attract natural enemies of the herbivore. Examples of this indirect form of defense are the emission of volatile organic compounds (VOCs), also known as herbivore-induced plant volatiles (HIPVs), or the secretion of extrafloral nectar (Turlings *et al.*, 1995; Dicke *et al.*, 1999; Heil & McKey, 2003). In these tritrophic interactions, predators and/or parasitoids are actively recruited by the plant to aid in its defense against the attacking herbivore.

Induced defense against microbial pathogens

The first event leading to activation of anti-microbial defenses is the recognition of the pathogen. It is commonly assumed that the majority of potential pathogens are recognized by microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs), such as flagellin, lipopolysaccharides, glycoproteins and chitin (Medzhitov & Janeway, 1997; Gomez-Gomez & Boller, 2000; Jones & Takemoto, 2004; Nürnberger *et al.*, 2004; Bittel & Robatzek, 2007). These microbial determinants activate pattern-recognition receptors (PRRs), which initiate a diversity of MAPK (MITOGEN-ACTIVATED PROTEIN KINASE)-dependent signaling events (Mészáros *et al.*, 2006) that ultimately result in the activation of PAMP-triggered immunity (PTI) (Jones & Dangl, 2006). Early responses, such as e.g. ethylene (ET) emission and reactive oxygen species (ROS) production, are remarkably similar upon perception of different PAMPs, suggesting an early point of convergence in the signaling pathway (He *et al.*, 2006). Recently, the receptor-like kinase BAK1 (BRASSINOSTEROID-ASSOCIATED KINASE 1) was identified as a potentially important regulator in this signaling convergence (Heese *et al.*, 2007). Furthermore, specific endogenous peptides that have recently been found to act as amplifiers in different disease signaling pathways might overrule specificity of the PAMP signal by activating a broad range of downstream genes (Huffaker & Ryan, 2007). Yet, every attacker seems to confer an adapted defense response, suggesting additional layers of regulation that specify the outcome of the plant's defense reaction (De Vos *et al.*, 2005).

Pre- and post-penetration resistance

Inducible defense mechanisms contributing to PTI can act at different stages of infection. A first line of defense acts against penetration of the host tissue (Collins *et al.*, 2003; Lipka *et al.*, 2005; Stein *et al.*, 2006). ABA appears to be a regulator of pre-invasion defenses. It mediates stomatal closure upon attack by a bacterial pathogen (Melotto *et al.*, 2006), thereby limiting further access. In addition, ABA enhances formation of callose-containing cell wall appositions at sites of attempted fungal or oomycetal entry in *Arabidopsis* (Ton & Mauch-Mani, 2004; Ton *et al.*, 2005; Kaliff *et al.*, 2007). In tomato, callose formation, which is at least in part ABA-dependent, contributes to basal resistance against the gray mold pathogen *Botrytis cinerea* (Asselbergh & Höfte, 2007).

A mutagenesis screen for *Arabidopsis* mutants impaired in penetration resistance to the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* revealed three novel regulatory genes. These so-called *PENETRATION* (*PEN*) genes *PEN1*, *PEN2* and *PEN3* encode a syntaxin protein, a glycosyl hydrolase and an ABC transporter, respectively (Collins *et al.*, 2003; Lipka *et al.*, 2005; Kobae *et al.*, 2006; Stein *et al.*, 2006). Given the nature of the proteins and the fact that they are recruited to plasma membrane domains directly underneath or in the vicinity of the site of pathogen entry, it is assumed that PEN proteins take part in vesicle-associated cell wall reinforcement and/or secretion of antimicrobial compounds (Collins *et al.*, 2003; Assaad *et al.*, 2004; Bonifacino & Glick, 2004; Bhat *et al.*, 2005; Lipka *et al.*, 2005; Stein *et al.*, 2006).

The importance of vesicle transport in penetration resistance is supported by reports showing that actin polymerization is essential for penetration resistance against fungi and oomycetes (Takemoto & Hardham, 2004; Hardham *et al.*, 2007). Targeted transport of defense-related vesicles and their fusion with the plasma membrane is regulated by syntaxins, such as PEN1, as well as other components of the SNARE (synaptosome-associated protein receptor) complex (Collins *et al.*, 2003; Pratelli *et al.*, 2004). The fusion of these vesicles with the plasma membrane enables the cell to deliver its antimicrobial cargo into the apoplast, using the ABC-transporters encoded by *PEN3* (Kobae *et al.*, 2006; Stein *et al.*, 2006). It is hypothesized that some of the antimicrobial compounds that contribute to penetration resistance are formed from non-toxic precursors through the activity of the glycosyl hydrolase PEN2 that is localized around the peroxisomes (Lipka *et al.*, 2005). A fourth protein, MLO2, (MILDEW RESISTANCE LOCUS O 2) has been implicated in penetration resistance in both barley and *Arabidopsis* (Jorgensen, 1992; Büschges *et al.*, 1997). *MLO2* encodes a plant-specific integral membrane protein containing seven transmembrane domains. Based on this structure and on their subcellular location, membrane topology, and domain-specific sequence, MLO proteins might function as G-protein-coupled receptors (Devoto *et al.*, 1999). The MLO2 protein seems to act antagonistically to PEN1 in promoting fungal ingress (Consonni *et al.*, 2006). Probably, MLO2 acts as a repressor of PEN-mediated resistance, thereby limiting the extent of subcellular changes and metabolic costs associated with penetration resistance.

Despite the sophisticated temporal and spatial regulation of penetration resistance, pathogens can successfully overcome this first layer of inducible defense. These micro-organisms face a second line of inducible defenses that targets growth and development of the pathogen (Lipka *et al.*, 2005). This level of basal resistance involves enhanced production and accumulation of proteins and metabolites with antimicrobial properties, such as PR- (PATHOGENESIS-RELATED) proteins and phytoalexins (Jackson & Taylor, 1996; Van Loon *et al.*, 2006b).

HORMONAL REGULATION OF INDUCED DEFENSES

Phytohormones play an important role in the regulation of post-penetration resistance. Particularly salicylic acid (SA) and jasmonic acid (JA) are recognized as key players in the regulation of induced defense against pathogens and insects (Pieterse & Van Loon, 1999; Glazebrook, 2001; Thomma *et al.*, 2001; Durrant & Dong, 2004; Pozo *et al.*, 2004; Schilmiller & Howe, 2005). Other hormones, such as ET, ABA, auxin, cytokinin, gibberellic acid, and brassinosteroids, have been shown to be important in the fine-tuning of SA- or JA-dependent resistance (Nakashita *et al.*, 2003; Goda *et al.*, 2004; Ton & Mauch-Mani, 2004; Mauch-Mani & Mauch, 2005; Navarro *et al.*, 2006; Siemens *et al.*, 2006; Van Loon *et al.*, 2006a; Adie *et al.*, 2007; Von Dahl & Baldwin, 2007; Wang *et al.*, 2007). For several years, it was commonly accepted that SA-inducible defenses were mostly effective against biotrophic pathogens, whereae JA/ET-dependent defenses were predominantly active against insects and necrotrophic pathogens (Glazebrook, 2001; Thomma *et al.*, 2001; Kessler & Baldwin, 2002; Schilmiller & Howe, 2005; Grant & Lamb, 2006). However, it has become clear that there are many exceptions to this notion (Thaler *et al.*, 2004; Stout *et al.*, 2006). In fact, plants react with a surprising specificity to attack by different pathogens or insects (Reymond & Farmer, 1998; Rojo *et al.*, 2003; De Vos *et al.*, 2005). In response to each of these attackers a highly specific blend of alarm signals is produced that varies in quantity, composition and timing. It is thought that this so-called "signal signature" contributes to the specificity of the plant's defense response (Thaler *et al.*, 2002b; De Vos *et al.*, 2005; Mur *et al.*, 2006).

SA-inducible defense signaling

The importance of SA in the regulation of induced defense became evident through experiments with transgenic NahG plants, that convert SA into catechol through the activity of salicylate hydroxylase encoded by the bacterial transgene *nahG*. Expression of this gene renders tobacco and *Arabidopsis* plants not only incapable of accumulating SA (Gaffney *et al.*, 1993), but also more susceptible to many pathogens, including bacteria, viruses, fungi and oomycetes (Delaney *et al.*, 1994; Kachroo *et al.*, 2000). Similarly, *Arabidopsis* mutants that are not able to enhance the production of SA upon pathogen infection, such as *eds1* (*enhanced disease susceptibility 1*), *sid1* (*salicylic acid induction-deficient 1*) (*eds5*), *sid2* (*eds16*), *pad4* (*phytoalexin-deficient 4*), and *gdg1* (*GH3-like defense gene 1*) display a higher level of susceptibility to these pathogens. Thus, SA is an important regulator of basal resistance against a wide spectrum of pathogens (Rogers & Ausubel, 1997; Zhou *et al.*, 1998; Nawrath & Métraux, 1999; Feys *et al.*, 2001; Wildermuth *et al.*, 2001; Jagadeeswaran *et al.*, 2007).

NPR1: a master regulator of SA-mediated defenses

Pathogen-induced accumulation of SA is required for many inducible defense reactions. The key regulatory protein NPR1 (NONEXPRESSOR OF PR-GENES 1) — also known as NIM1

(NON IMMUNITY 1) or SAI1 (SALICYLIC ACID-INSENSITIVE 1) — plays a critical role in this SA-dependent signaling pathway. Mutations in the *NPR1* gene render the plant largely unresponsive to pathogen-induced SA production, thereby blocking the induction of SA-dependent *PR* genes (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). NPR1 is expressed throughout the plant at low levels and shows only modest induction upon infection or SA treatment in wild-type *Arabidopsis* (Cao *et al.*, 1997; Ryals *et al.*, 1997). Overexpression of *NPR1* does not result in a massive induction of the marker gene *PR-1*, indicating that NPR1 requires post-translational activation in order to transduce the SA signal (Cao *et al.*, 1998; Friedrich *et al.*, 2001). Indeed, SA-induced redox changes have been shown to reduce intermolecular disulfide bonds that hold NPR1 together in an inactive oligomer complex. This reduction releases monomeric NPR1, which is translocated into the nucleus (Mou *et al.*, 2003), where it interacts with different proteins, such as TGA transcription factors (TFs) and NIMINs (NIM1-INTERACTING), and modulates the expression of downstream genes, such as *PR-1* (Weigel *et al.*, 2001; Fan & Dong, 2002; Weigel *et al.*, 2005; Kesarwani *et al.*, 2007).

Recently, a genomics-directed approach demonstrated that a select group of WRKY TFs is induced upon nuclear translocation of NPR1 monomers (Wang *et al.*, 2006). These WRKY TFs can have positive or negative effects on the inducible expression of *PR* genes, thereby further contributing to the complexity of the SA- and NPR1-dependent signaling network (Wang *et al.*, 2006; Eulgem & Somssich, 2007). In addition to regulating *PR* genes, NPR1 has been shown to target the transcription of genes that are involved in protein folding, modification and secretion. Expression of these proteins ensures a proper processing of *PR*-transcripts and secretion of PR-proteins, which contributes to SA-based resistance (Wang *et al.*, 2005).

Although the NPR1 protein is a key regulator of SA-inducible plant responses, other reports have described SA-inducible defense reactions that do not require NPR1 (Uquillas *et al.*, 2004; Blanco *et al.*, 2005). For example, early SA-responsive genes, such as *GLUTATHIONE S-TRANSFERASE* (*GST6*) and *GLUCOSYLTRANSFERASE* (*EIGT*), have been reported to be induced in *npr1-1* plants upon treatment with SA (Uquillas *et al.*, 2004). Furthermore, several gain-of-function mutations in *Arabidopsis* were found to confer elevated levels of SA-inducible gene expression in the *npr1* mutant background, such as *snc1* (*suppressor of npr1-1, constitutive 1*) (Li *et al.*, 2001), *ssi1* *(suppressor of sa insensitivity 1)* (Shah *et al.*, 1999), *ssi2* (Shah *et al.*, 2001), *cpr5* *(constitutive expresser of pr genes 5)* (Bowling *et al.*, 1997), *cpr6* (Clarke *et al.*, 1998), and *hrl1* *(hypersensitive reponse-like lesions 1)* (Devadas *et al.*, 2002).

Potential downstream factors in the regulation of these SA-inducible, NPR1-independent plant responses are WHIRLY (WHY) proteins. Members of this relatively small family of transcription factors can form tetrameric structures that bind to single-stranded DNA sequences (Desveaux *et al.*, 2002). The ssDNA-binding activity of AtWHY1 to promoters of *PR*-genes is induced by SA independently of NPR1 (Desveaux *et al.*, 2005). Furthermore, mutations of AtWHY1 in its DNA-binding domain were found to affect SA-induced *PR-1* expression and resistance to *Hyaloperonospora parasitica*.

Together, these results suggest that AtWHY1 works in conjunction with NPR1 to activate SA-dependent defenses (Desveaux *et al.*, 2005).

Negative regulation of SA-mediated defenses

The activation of SA-inducible defensive mechanisms is associated with metabolic costs due to the allocation of limited resources to defensive compounds, or to toxicity of the induced defenses to the plant's own metabolism (Heil, 2002; Walters & Boyle, 2005; Van Hulten *et al.*, 2006). It is, therefore, essential that plants tightly regulate SA-induced responses, and prevent excessive activation. The PMR4 (POWDERY MILDEW RESISTANT 4) protein has been demonstrated to suppress SA-inducible defense mechanisms (Nishimura *et al.*, 2003). As part of a collection of *Arabidopsis* mutants resistant to the powdery mildew fungus *Erysiphe cichoracearum* (Vogel & Somerville, 2000), the *pmr4* mutant was used to map the gene responsible for the increased resistance. Surprisingly, *PMR4* was found to encode a stress-induced callose synthase (Nishimura *et al.*, 2003). As described above, formation of callose-containing cell wall appositions (papillae) is commonly considered to contribute to disease resistance (Ton & Mauch-Mani, 2004; Asselbergh & Höfte, 2007). The fact that *pmr4* showed enhanced resistance rather than enhanced susceptibility resulted from elevated SA-levels (Nishimura *et al.*, 2003) in this mutant. This suggests that PMR4 not only functions as a positive regulator of penetration resistance, but also plays a role in suppression of SA-dependent defenses. Hence, inducible defense responses can counteract each other, possibly to limit defense to the tissue attacked.

An additional layer of negative regulation of SA-inducible defenses is controlled by the redox-buffering capacity of the cell. As mentioned earlier, SA-induced redox change is required to release monomeric NPR1 (Mou *et al.*, 2003). Stabilization of this redox change by increased levels of glutathione suppresses monomerization and translocation of NPR1 to the nucleus. As a result, downstream SA-dependent defenses are no longer activated (Mou *et al.*, 2003). Also at the transcriptional level regulatory proteins operate that prevent excessive activation of SA-induced responses. For instance, NIMINs and SNI1 (SUPPRESSOR OF NPR1 INDUCIBLE 1) counteract NPR1 action at this level (Weigel *et al.*, 2005; Mosher *et al.*, 2006; Kesarwani *et al.*, 2007). Thus, in concerted action with positive regulators of SA-dependent defenses, these inhibitory mechanisms allow the plant to fine-tune its defensive reaction.

JA-dependent defense signaling

JA and its functionally active derivatives methyl JA (MeJA) and JA-isoleucine (JA-Ile), are produced by the octadecanoid pathway after stimulation by pathogens or insects. Downstream target genes include defense-related genes, such as the defensin *PDF1.2* (*PLANT DEFENSIN 1.2*) and thionin *THI2.1* (*THIONIN 2.1*), but also genes that are required for the biosynthesis of JA, providing a feed-forward loop. The central role for JA in plant resistance became clear when different research groups showed that mutants defective

in either the biosynthesis of, or responsiveness to, JA displayed an enhanced disease susceptibility to certain pathogens or insects (Creelman & Mullet, 1997; Pozo *et al.*, 2004; Devoto & Turner, 2005; Wasternack, 2007). For example, the *Arabidopsis fad3fad7fad8* (*fatty acid desaturation 3, 7, 8*) JA-biosynthesis triple mutant showed susceptibility to normally non-pathogenic *Pythium* spp. (Staswick *et al.*, 1998; Vijayan *et al.*, 1998), enhanced disease severity due to cucumber mosaic virus (Ryu *et al.*, 2004b), and high mortality from attack by larvae of the fungal gnat (*Bradysia impatiens*) (McConn *et al.*, 1997). Similarly, the JA-insensitive *coi1* (*coronatine insensitive 1*) mutant proved to be enhanced susceptible to the bacterial leaf pathogen *Erwinia carotovora* (Norman-Setterblad *et al.*, 2000) and the necrotrophic fungi *Alternaria brassicicola* and *B. cinerea* (Thomma *et al.*, 1998).

All plant responses to JA described so far in *Arabidopsis*, are dependent on the COI1 protein (Feys *et al.*, 1994; Devoto & Turner, 2003), which, in a complex with JAZ (JASMONATE ZIM-DOMAIN) proteins, functions as the receptor of the active JA-amino acid conjugate JA-Ile (Chini *et al.*, 2007; Thines *et al.*, 2007). COI1 encodes an F-box protein (Xie *et al.*, 1998), which is part of an SCF^{COI1} E3 ubiquitin ligase complex that is involved in proteasome-mediated protein degradation (Devoto *et al.*, 2002; Xu *et al.*, 2002). The F-box protein confers specificity to the E3 ligase complex by interacting with proteins that are targeted for ubiquitination and subsequent degradation. Therefore, COI1 is thought to act through the removal of repressors of JA-responsive genes (Devoto *et al.*, 2003). Recently, the JAZ proteins were identified as likely candidates (Chini *et al.*, 2007; Thines *et al.*, 2007): JAZ proteins repress JA-responsive genes by actively suppressing transcriptional activators of JA-responsive genes, such as *MYC2*. Upon stimulation by JA, the physical interaction of JA-Ile with JAZ proteins allows COI1 to target the latter for degradation by the proteasome. As a result, the repression by the JAZ proteins is lifted, resulting in enhanced transcription of JA-responsive genes. JAZ genes are themselves induced by JA, indicating a negative feedback loop that allows for termination of the JA response.

Cross-talk between defense signaling pathways

The signaling pathways that are controlled by SA and JA regulate different defense responses that are effective against partially distinct classes of attackers. Cross-communication between the two pathways has been put forward as an additional mechanism by which plants fine-tune their defense responses (Reymond & Farmer, 1998; Pieterse & Van Loon, 1999; Rojo *et al.*, 2003; Bostock, 2005; Beckers & Spoel, 2006). Most reports indicate a mutually antagonistic interaction between SA- and JA-dependent signaling (Bostock, 2005). As a result of this negative cross-talk, activation of the SA response should render a plant more susceptible to JA-resisted necrotrophs and herbivorous insects, and vice versa. Indeed, trade-offs between SA-dependent resistance against biotrophic pathogens, on the one hand, and JA-dependent defense against

necrotrophic pathogens or insect herbivores, on the other hand, have been reported (Felton & Korth, 2000; Bostock, 2005; Pieterse & Dicke, 2007; Koornneef & Pieterse, *submitted*). However, synergistic actions of SA and JA in plant defense have been described as well (Schenk *et al.*, 2000; Van Wees *et al.*, 2000; Mur *et al.*, 2006; Truman *et al.*, 2007).

Over the past years, various regulatory components have been identified in the cross-talk between SA- and JA-dependent signaling pathways (Kachroo *et al.*, 2003; Spoel *et al.*, 2003; Li *et al.*, 2004; Brodersen *et al.*, 2006; Ndamukong *et al.*, 2007). These include proteins with stimulatory and repressive functions in both SA-dependent and JA-dependent responses. One of the key regulators in cross-communication between SA and JA signaling is NPR1, as mutants in this protein are impaired in the SA-induced suppression of JA-inducible *PDF1.2* expression (Spoel *et al.*, 2003; Yuan *et al.*, 2007). Hence, NPR1 not only plays a role in the induction of SA-inducible genes, but is also required to prioritize SA-dependent responses over JA-dependent responses. Notably, the function of NPR1 in cross-talk between SA and JA signaling does not require nuclear localization, suggesting also a cytosolic mode of action by this regulatory protein (Spoel *et al.*, 2003; Yuan *et al.*, 2007).

Other hormones have been shown to influence the cross-talk between SA- and JA-defense signaling pathways. For example, ET and ABA antagonistically influence JA-inducible defense mechanisms against pathogens and insects through MYC2 and ERF1 (ET RESPONSE FACTOR 1). These transcription factors regulate divergent branches of the JA-signaling route that are involved in the response to wounding and pathogen attack, respectively. In response to ABA and JA, MYC2 induces the expression of genes such as *VSP2* (*VEGETATIVE STORAGE PROTEIN 2*) and *LOX2* (*LIPOXYGENASE 2*). At the same time, it represses genes that are responsive to JA and ET, amongst which *PDF1.2*. In contrast, ERF1 positively controls JA- and ET-responsive genes, while it attenuates the expression of genes that are responsive to JA and ABA (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004). Recent observations (Pré, 2006) suggest that ORA59 (OCTADECANOID-RESPONSIVE *ARABIDOPSIS* AP2/ERF 59), rather than ERF1, is the key TF in the integration of JA and ET signaling. In *ORA59*-silenced plants, ERF1 was no longer able to induce *PDF1.2* gene expression in response to JA or ET, or upon infection with the necrotrophic fungi *B. cinerea* or *A. brassicicola*. Furthermore, the *ORA59*-silenced lines were more susceptible to *B. cinerea*, despite expressing wild-type levels of *ERF1*.

ABA not only influences JA-signaling through MYC2, but also negatively affects SA-dependent defense responses (Audenaert *et al.*, 2002; Thaler & Bostock, 2004; Mauch-Mani & Mauch, 2005). For example, *Pseudomonas syringae* pv. *tomato* infection was shown to upregulate ABA signaling, thereby increasing plant susceptibility to this pathogen (De Torres-Zabala *et al.*, 2007; Mohr & Cahill, 2007). Conversely, ERD15 (EARLY RESPONSE TO DEHYDRATION 15) was identified as a negative regulator of ABA signal transduction, and positively influenced SA-responsive gene expression and resistance to the bacterial necrotroph *E. carotovora* pv. *carotovora* (Kariola *et al.*, 2006).

SUPPRESSION OF BASAL RESISTANCE BY PATHOGENS AND INSECTS

During the evolutionary arms-race between plants and their attackers, the latter group of organisms found mechanisms to circumvent, or even suppress plant defense mechanisms. For instance, phloem-sucking aphids have been shown to prevent sieve tube plugging, allowing optimal exploitation of the hosts' carbohydrates (Will *et al.*, 2007). Caterpillars of the cotton bollworm (*Helicoverpa zea*; also known as corn earworm or tomato fruitworm) and larvae of the diamondback moth (*Plutella xylostella*) can disarm the plant's weaponry by reducing nicotine production and deactivating a generally very effective two-component defense-mechanism known as the "mustard oil bomb", respectively (Musser *et al.*, 2002; Ratzka *et al.*, 2002). Bacteria achieve suppression of defense by injecting virulence factors into host cells that counteract PTI (Alfano & Collmer, 1997; Jones & Dangl, 2006). Most of these effector molecules inactivate signal transduction cascades that are required for the induction of defense (Abramovitch *et al.*, 2006; Chisholm *et al.*, 2006; Grant *et al.*, 2006; De Torres-Zabala *et al.*, 2007; Fu *et al.*, 2007; He *et al.*, 2007; Shan *et al.*, 2007), while others disable the production of signaling hormones (Jelenska *et al.*, 2007). Analogous to the situation with bacterial pathogens, fungi and oomycetes likewise repress host defense reactions through the secretion of virulence factors. For instance, the effector AVR2 of *Cladosporium fulvum* inhibits tomato RCR3, an extracellular cysteine protease that is required for activation of *C. fulvum*-induced defense responses (Rooney *et al.*, 2005). Similarly, two protease inhibitors of *Phytophthora infestans* have been demonstrated to repress the activity of tomato pathogenesis-related P69B-like proteases (PR-7) (Tian *et al.*, 2004; 2005).

Mimicking plant hormones to promote disease

Some microbial pathogens have acquired the ability to manipulate the plant's defense signaling by producing phytohormones or functional mimics thereof to "trick" the plant into activating inappropriate defenses (Robert-Seilaniantz *et al.*, 2007). For instance, coronatine is produced by virulent *P. syringae* strains and functions as an extremely potent mimic of JA-Ile (Nomura *et al.*, 2005). Coronatine acts as JA-Ile in suppressing SA-dependent defenses through pathway cross-talk (Zhao *et al.*, 2003; Brooks *et al.*, 2005; Cui *et al.*, 2005; Laurie-Berry *et al.*, 2006). Recently, coronatine has also been demonstrated to suppress PAMP-induced stomatal closure, thereby facilitating entry of the bacteria into the leaf (Melotto *et al.*, 2006; Underwood *et al.*, 2007).

Many bacterial and fungal pathogens are able to produce auxin (Glickmann *et al.*, 1998; Maor *et al.*, 2004; Valls *et al.*, 2006), which has been shown to enhance disease susceptibility (Robinette & Matthysse, 1990; Navarro *et al.*, 2006; Wang *et al.*, 2007). Consequently, disabling auxin production by the pathogen decreased its virulence in some of the interactions studied (Robinette & Matthysse, 1990; Valls *et al.*, 2006). Production of gibberellic acid, cytokinin and ABA have also been reported for multiple

plant pathogens (Candau *et al.*, 1992; Jameson, 2000; Walters & McRoberts, 2006). All of these hormones are known to influence disease resistance pathways through different cross-talk mechanisms (Mauch-Mani & Mauch, 2005; Walters & McRoberts, 2006; Robert-Seilaniantz *et al.*, 2007).

Like pathogens, insects have the ability to induce ineffective plant signaling cascades as a decoy mechanism. Silverleaf whitefly (*Bemisia tabaci*) nymphs trigger SA-responsive gene expression and, as a consequence, suppress the induction of JA-dependent defenses that are effective against this insect (Kempema *et al.*, 2007; Zarate *et al.*, 2007). Hence, silverleaf whitefly nymphs are capable of exploiting the negative cross-talk between SA and JA to make the plant more accessible to infestation (Zarate *et al.*, 2007). Recently, also egg-derived elicitors from the large cabbage white (*Pieris brassicae*) and the small cabbage white (*Pieris rapae*) have been suggested to suppress JA-dependent responses through SA-induced cross-talk (Little *et al.*, 2007). Apparently, cross-talk mechanisms in plants are commonly exploited by herbivorous insects to interfere with the defense reactions of their hosts.

COUNTERACTING RESISTANCE SUPPRESSION

Taken together, it seems evident that suppression of plant defense mechanisms is a common strategy amongst plant attackers. The ability to decoy phytohormone or PAMP-signaling seems indispensable for a pathogen or herbivorous insect to be successful. In response, plants have evolved mechanisms to counteract these resistance-suppressing abilities of pathogens and insects (Shang *et al.*, 2006). For instance, to attenuate auxin-mediated disease susceptibility, plants are equipped with a microRNA (miRNA)-based defense response mechanism that is turned on upon PAMP detection (Navarro *et al.*, 2006). Perception of the bacterial PAMP flagellin triggers the production of miR393s that block the formation of F-box auxin receptors. Analogous to the situation in JA-signaling, activated F-box receptors target transcriptional inhibitors of AUX-responsive genes for degradation by the proteasome (Gray *et al.*, 2001), thereby lifting the transcriptional repression (Dharmasiri & Estelle, 2004). As miR393s hamper the formation of F-box proteins, they prevent activation of the AUX-response and the subsequent increase in susceptibility (Navarro *et al.*, 2006).

Many plants produce R- (RESISTANCE) proteins by which they recognize, directly or indirectly, specific effector proteins that act as virulence factors (Dangl & Jones, 2001). As a result of this recognition, a wide array of defenses are activated faster and to a higher extent than in PTI (Tao *et al.*, 2003; Truman *et al.*, 2006; Shen *et al.*, 2007). This phenomenon is known as *R*-gene-mediated resistance, also referred to as gene-for-gene resistance, or effector-triggered immunity (ETI) (Kim *et al.*, 2005; Jones & Dangl, 2006). In the absence of a cognate R-protein, effectors target a defensive mechanism in order to suppress it. Due to presence of the R-protein, the target is guarded and suppression is prevented: virulence turns to avirulence. Often, effector recognition leads to localized

cell death, a defense mechanism known as the hypersensitive response (HR). Throug the HR, the plant can inhibit further growth of the pathogen by sacrificing the cells that were initially attacked (Greenberg *et al.*, 1994). Not surprisingly, ETI is most effective against pathogens with a (hemi-) biotrophic life-style (Glazebrook, 2005; Jones & Dangl, 2006).

ETI is extremely effective against otherwise virulent pathogens. A major limitation of this form of resistance is that it is operative only against races of the pathogen that secrete an effector protein that is complementary to one of the R-proteins expressed by the plant. Because of selection pressure on the pathogen, variation in effector effectiveness can overcome plant resistance: mutants of the pathogen that secrete a structurally modified effector protein are no longer recognized by the host. As a result, these mutants are able to effectively colonize the host tissue, have a higher reproductive success, and may eventually become the dominant race of the pathogen. In return, evolutionary selection pressure favors mutated forms of plant R-proteins that are able to recognize effector proteins of the mutated pathogen. Interaction between an altered R-protein with the dominant effector of the pathogen will again activate ETI. This perpetual process of selection and recognition, alternating between pathogens and their hosts, is reflected in the denominator gene-for-gene resistance.

INDUCED RESISTANCE: ENHANCING THE PLANT'S DEFENSIVE CAPACITY

A very different strategy is one in which plants augment their defensive potential in the face of repeated attack. Upon exposure to mild biotic stress, they are capable of acquiring an enhanced level of resistance that is effective against future attack by a broad range of pathogens and insects. This form of plant defense is commonly referred to as "induced resistance" (Van Loon, 2000). Over the past three decades, distinct forms of induced resistance have been identified and defined on the basis of differences in the signal-transduction pathways involved. Induced resistance can be activated by microbial pathogens and insect herbivores, but also by beneficial micro-organisms, such as mycorrhizal fungi and plant growth-promoting rhizobacteria (Kessler & Baldwin, 2002; Dicke & Hilker, 2003; Pozo *et al.*, 2004). While ETI is directed specifically against the microbial invader encountered, induced resistance is typically characterized by a broad spectrum of effectiveness (Kuc, 1982). Moreover, induced resistance often acts systemically in plant parts distant from the site of primary attack, thereby protecting the entire plant against subsequent invaders.

Several biologically induced, systemic defense responses have been characterized in detail: 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); and wound-induced resistance (WIR), which is typically elicited upon tissue damage, such as caused by chewing insects (Kessler & Baldwin, 2002; Howe, 2004). In addition, broad-spectrum resistance can be induced by chemicals such as the non-protein amino-acid ß-amino butyric acid (BABA) (Zimmerli *et al.*, 2000). An overview of the spectrum of effectiveness of these four types of induced resistance is presented in Figure 1.1.

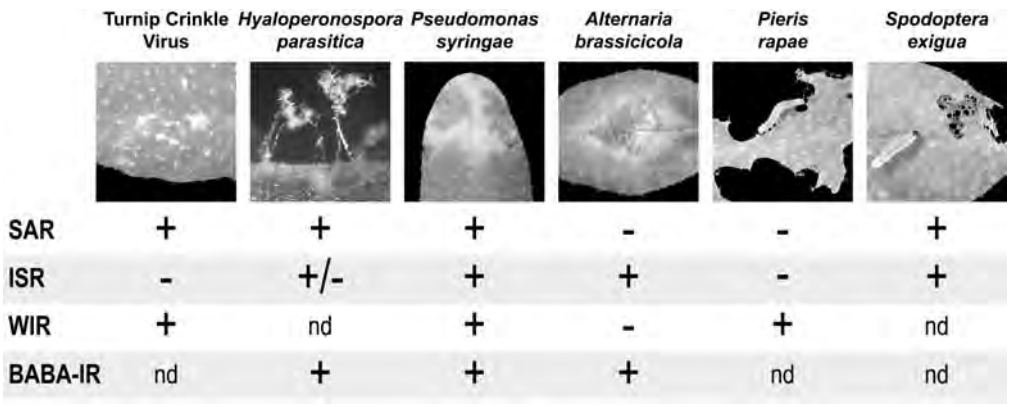

Figure 1.1. Spectrum of effectiveness of systemically induced resistance in *Arabidopsis thaliana*. **Effectiveness is indicated by +, ineffectiveness by – (nd; not determined). SAR is effective against (hemi-) biotrophic pathogens such as TCV, *H. parasitica*, and *P. syringae* (Ton *et al.*, 2002b). By contrast, ISR is also effective against necrotrophic pathogens such as *A. brassicicola* (Ton *et al.*, 2002b). Both SAR and ISR are effective against the generalist herbivore *S. exigua* whereas the specialist herbivore *P. rapae* is unaffected by both induced resistance responses (Van Oosten, 2007). WIR, induced by *P. rapae* caterpillars, confers resistance against subsequent infestation by *P. rapae* and against TCV and *P. syringae*, but not against *A. brassicicola* (De Vos *et al.*, 2006b). BABA-IR is effective against all attackers shown here (Ton & Mauch-Mani, 2004) .**

SYSTEMIC ACQUIRED RESISTANCE

Systemic acquired resistance (SAR) is the classic form of induced resistance and develops in non-infected tissues upon a primary infection with a necrosis-inducing pathogen (Ross, 1961). SAR depends on the plant's ability to produce and perceive SA (White, 1979; Malamy *et al.*, 1990; Métraux *et al.*, 1990; Ward *et al.*, 1991; Uknes *et al.*, 1992). Avirulent pathogens that activate ETI resulting in an HR are potent inducers of SAR. However, PTI, when activated by PAMPs that induce the SA signaling pathway, can trigger SAR as well (Mishina & Zeier, 2007). Like other induced resistance phenomena, pathogen-induced SAR is effective against a broad range of pathogens, although it seems predominantly effective against pathogens with a (hemi-) biotrophic lifestyle (Ton *et al.*, 2002b).

SAR signal transduction

Studies with transgenics and mutants that are impaired in SA production or responsiveness have revealed that SA is central to the induction and expression of SAR (Gaffney *et al.*, 1993; Lawton *et al.*, 1995; Nawrath & Métraux, 1999). However, for a long time it remained unclear whether this molecule functions as the systemically transported signal. Grafting experiments with wild-type and SA-non-accumulating NahG tobacco demonstrated that SA production is not required for the generation of the mobile signal in SAR (Vernooij *et al.*, 1994). On the other hand, Shulaev *et al.* (1995) and Molders *et al.* (1996) demonstrated that radioactively labelled SA, synthesized at the site of primary infection, is transported throughout tobacco and cucumber plants, respectively. Seskar *et al.* (1998) proposed that methyl salicylate (MeSA) is synthesized from SA in the locally infected leaves and acts in the systemic target tissues by being converted back into SA. This hypothesis is supported by later findings that SAMT (SA METHYL TRANSFERASE) and the MeSA esterase SABP2 (SA-BINDING PROTEIN 2) are essential for the expression of SAR in locally infected and systemic leaves, respectively (Forouhar et al., 2005; Kumar and Klessig, 2003; Park et al., 2007). Verberne *et al.* (2003) clearly demonstrated that in tobacco ET-perception is required locally to allow generation of SAR to tobacco mosaic virus (TMV). Wild-type scions that were grafted onto ET-insensitive rootstocks did not show build-up of SA or *PR*-transcripts when the rootstocks were inoculated with TMV. Locally, SA production and *PR*-gene expression were not affected, indicating a role for ET in the generation or transport of the SAR signal. Possibly, ET perception is required for SAMT activity. Further experiments are needed to elucidate the relationship between ET and SAR.

Besides SA and MeSA, lipid-derived components have been implicated as systemic signals in SAR as well. In a screen for *Arabidopsis* mutants defective in biologically induced SAR against *P. syringae* and *H. parasitica*, Maldonado *et al.* (2002) identified the *dir1 (defective in induced resistance 1)* mutant. This mutant carries a mutation in a putative apoplastic lipid transfer protein (LTP). *In vitro* LTPs have been demonstrated to bind and subsequently transfer lipids between membranes. Therefore, the DIR1 LTP might be required to transport a lipid-derived compound. Phenotypic characterization of the mutant revealed that *dir1* plants are not impaired in the activation of local PTI and ETI, but only in the systemic transmission of the SAR-inducing signal. DIR1 was demonstrated to act upstream of NPR1 and, therefore, could be involved in promotion of long-distance signaling. Remarkably, *dir1* still accumulated enhanced levels of SA in the distal leaves after induction treatment with an avirulent strain of *P. syringae*. Analogous to its agonistic function during ETI (Shirasu *et al.*, 1997), the role of SA in biologically induced SAR might be to amplify a DIR-dependent SAR signal.

Like *dir1*, *sfd1 (suppressor of fatty acid desaturase deficiency 1)* mutants are also attenuated specifically in the activation of SAR (Nandi *et al.*, 2004). This phenotype results from the mutant's inability to accumulate SA in distal tissues. However, *sfd1* is not disrupted in SA metabolism but rather in the synthesis of plastidic glycerolipids, which again suggests a role for lipid-derived compounds in SAR. Truman *et al.* (2007)

suggested a central role for JAs in SAR signaling. Since these molecules are derived principally from the fatty acid linolenic acid, JAs are also lipid-derived compounds. Although JA- and SA-signaling pathways are mostly antagonistic (Bostock, 2005), different lines of evidence point to a possible role for JA in early stages of SAR. Firstly, analyses of gene expression and hormone accumulation in systemic parts of biologically induced SAR-expressing plants demonstrated an induction of JA-biosynthesis and –responsive genes, and an increase in JA content, respectively. Secondly, mutants in JA-signaling *sgt1b (suppressor of g2 allele of SKP1* (SUPPRESSOR OF KINETOCHORE PROTEIN 1) *1b)*, *opr3* (*12-oxo-phytodienoate reductase 3*) and *jin1 (jasmonate insensitive 1)*, failed to develop SAR upon leaf infiltration with avirulent *P. syringae* pv. *tomato*. However, other mutants that are disrupted in JA-signaling, namely *jar1 (jasmonate resistant 1)* and *eds8*, are still able to show biologically activated SAR (Pieterse *et al.*, 1998; Ton *et al.*, 2002a). Therefore, the exact role of JA-signaling in SAR needs to be further explored.

Recently, a novel signaling component in SAR was identified. Mishina & Zeier (2006) demonstrated in *Arabidopsis* that FMO1 (FLAVIN-DEPENDENT MONOOXYGENASE 1) plays a critical role in the onset of SAR. Transcription of the *FMO1* gene was induced locally and systemically upon inoculation with an avirulent strain of *P. syringae* pv. *tomato*. Analysis of a T-DNA knockout mutant revealed that FMO1 is important in the amplification of the SAR signal in the systemic tissues (Mishina & Zeier, 2006). FMO1 also plays a role in basal resistance against *H. parasitica* and *P. syringae* (Bartsch *et al.*, 2006; Koch *et al.*, 2006). Bratsch *et al.* (2006) demonstrated that FMO1 mediates a SA-independent branch of EDS1 signaling. EDS1 has also been implicated in SAR signaling (Truman *et al.*, 2007), but Mishina & Zeier (2006) showed that *eds1* plants are still capable of establishing a SAR response. Hence, the role of EDS1 in SAR needs further study.

INDUCED SYSTEMIC RESISTANCE

Besides pathogens, non-pathogenic organisms can also elevate the level of basal resistance in plants. For instance, root colonization by selective strains of non-pathogenic *Pseudomonas* bacteria triggers an induced systemic resistance (ISR) response that is effective against diverse pathogens (Van Loon *et al.*, 1998). By performing bioassays with *Arabidopsis* mutants in different hormone response pathways, Pieterse *et al.* (1996; 1998) demonstrated that the signaling cascades underlying SAR and ISR are clearly distinct.

Systemic effects of biological control agents

Many non-pathogenic soil bacteria can protect plants against attack by harmful micro-organisms through competition for nutrients or secretion of antibiotics, biosurfactants or lytic enzymes (Van Loon *et al.*, 1998; Weller *et al.*, 2002; Harman *et al.*, 2004a; De Bruijn *et al.*, 2007; Perneel *et al.*, 2007). Fluorescent *Pseudomonas* spp. are among the

most effective rhizobacteria with biocontrol activity, 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; Perneel *et al.*, 2007).

Direct antagonistic activity of soil-borne pathogens was originally thought to be the sole mode of action of these biocontrol agents. However, some rhizobacterial strains are also capable of reducing disease in above-ground plant parts through ISR (Van Loon *et al.*, 1998). This was first evidenced by experiments in which the non-pathogenic rhizobacteria remained spatially separated from the pathogen, while disease symptoms were reduced (Van Peer *et al.*, 1991; Wei *et al.*, 1991). Like SAR, rhizobacteria-mediated ISR has been demonstrated in many plant species and is effective against a broad spectrum of plant attackers, including fungi, bacteria, viruses and even insects (De Vleesschauwer *et al.*, 2006; Van Loon & Bakker, 2006; Van Oosten, 2007). While SAR is predominantly operative against biotrophic pathogens that are resisted through SA-dependent defenses, ISR also functions against necrotrophic pathogens that are susceptible to JA-dependent defense responses, such as *A. brassicicola* (Ton *et al.*, 2002b). Over the last decade, it has become clear, that also other non-pathogenic micro-organisms are able to trigger systemically induced resistance, such as the endophytic fungus *Piriformospora indica* (Waller *et al.*, 2005), non-pathogenic strains of *Fusarium* spp. (Duijff *et al.*, 1998), *Trichoderma* spp. (Yedidia *et al.*, 2003; Shoresh *et al.*, 2005), *Penicillium* spp. (De Cal *et al.*, 2000; Koike *et al.*, 2001), and mycorrhizal fungi (Pozo *et al.*, 2002; 2004).

Mycorrhizal fungi are capable of forming an external or internal symbiosis with roots of the majority of plant species. While these fungi use plant-derived sugars as a nutrient source, they provide the host with an enhanced root surface to absorb limiting nutrients, such as phosphate (Harrison, 2005; Karandashov & Bucher, 2005). In addition, they have been shown to also enhance plant resistance against abiotic and biotic stress (Cordier *et al.*, 1998; Liu *et al.*, 2007; Pozo & Azcon-Aguilar, 2007).

Onset of ISR

The ability of plants to express ISR depends on the plant-rhizobacterium combination, which suggests a complex interaction between both organisms (Pieterse *et al.*, 2002). For instance, *Pseudomonas putida* WCS358r is capable of inducing ISR in *Arabidopsis*, but not in radish (Van Peer *et al.*, 1991; Van Peer & Schippers, 1992; Leeman *et al.*, 1995; Van Wees *et al.*, 1997) while, conversely, radish is responsive to *Pseudomonas fluorescens* WCS374r whereas *Arabidopsis* is not (Leeman *et al.*, 1995; Van Wees *et al.*, 1997). *Pseudomonas fluorescens* WCS417r (WCS417r) is capable of inducing ISR in both *Arabidopsis* and radish (Leeman *et al.*, 1995; Van Wees *et al.*, 1997), as well as in other species, e.g. carnation (Van Peer *et al.*, 1991), tomato (Duijff *et al.*, 1998), and bean (Bigirimana & Höfte, 2002), but not in *Eucalyptus* (Ran *et al.*, 2005) or rice (De Vleesschauwer *et al.*, 2006). Besides inter-species differences in ISR-inducibility, intra-

species variation is also observed. Most *Arabidopsis* accessions tested, including 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). These latter accessions are compromised in a common trait governing a step between the recognition of the bacterium and the expression of ISR, as all offspring resulting from a cross between Ws-0 and RLD1 were disrupted in the generation of ISR (Ton *et al.*, 1999). These data clearly demonstrate that the ability to express ISR is genetically determined.

Although conclusive evidence is still lacking, the striking homologies with sensitive perception mechanisms for pathogen-derived PAMPs that function in PTI, suggest that non-pathogenic rhizobacteria are recognized similarly. So far, several bacterially derived MAMPs have been implicated in the elicitation of rhizobacteria-mediated ISR. Examples are flagella, cell wall components, such as lipopolysaccharides, and secreted metabolites, such as siderophores and antibiotics (Kloepper *et al.*, 2004; Bakker *et al.*, 2007). However, often bacterial mutants lacking one of these determinants are still able to trigger ISR (Bakker *et al.*, 2003; Meziane *et al.*, 2005). This indicates that plants recognize multiple determinants produced by the same bacterial strain. Moreover, the fact that these MAMPs trigger ISR in many, but not all, plant species or genotypes, suggests that perception by MAMP-receptors differs between plant species and/or genotypes (Robatzek *et al.*, 2007).

The first indications that SAR and ISR are regulated differently came from the observation that WCS417r bacteria induced systemic resistance in radish without a concomitant accumulation of PR-proteins (Hoffland *et al.*, 1995). Similarly, WCS417r-mediated enhanced resistance in *Arabidopsis* against *F. oxysporum* f.sp. *raphani* and *P. syringae* pv. *tomato* did not coincide with activation of SAR-marker genes encoding PR-1, PR-2, and PR-5 (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Further support for the hypothesis that ISR is regulated by a signaling pathway physiologically different from SAR, arose from experiments demonstrating that, in contrast to SAR, 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). 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), cucumber (Press *et al.*, 1997), and tomato (Yan *et al.*, 2002). This wide range of induction of ISR indicates that the ability of rhizobacteria to activate an SA-independent pathway controlling systemic resistance is common to a broad range of plants.

ISR signal transduction

The non-involvement of SA in ISR prompted research to investigate a possible role of JA or ET in ISR signaling, because these hormones had also been reported to be involved in

induced defense responses (Farmer & Ryan, 1992; Penninckx *et al.*, 1996). Therefore, *Arabidopsis* mutants impaired in JA or ET responsiveness were tested. As it turned out, both *jar1* and *etr1* (*ethylene response 1*) (Bleecker *et al.*, 1988) were compromised in the expression of ISR in response to elicitation by WCS417r (WCS417r-ISR) against *P. syringae* pv. *tomato* (Pieterse *et al.*, 1998), indicating a requirement for JA and ET in ISR signaling. Similar results were obtained with other mutants in JA and ET signal transduction. *Arabidopsis* mutant *eds8*, which had been identified as having enhanced susceptibility to *P. syringae* pv. *maculicola* (Glazebrook *et al.*, 1996) was impaired in both WCS417r-ISR (Ton *et al.*, 2002a) and JA signaling (Ton *et al.*, 2002b; Glazebrook *et al.*, 2003). Furthermore, of a large set of well-characterized ET signaling mutants tested, none showed enhanced levels of resistance against *P. syringae* pv. *tomato* after root colonization by WCS417r (Knoester *et al.*, 1999). Although both JA and ET are required for ISR, the levels of these hormone were not changed upon colonization of *Arabidopsis* roots by WCS417r (Pieterse *et al.*, 2000). Thus, elicitation of ISR appears to sensitize systemic tissues for the perception of these hormones. In line with the differences in effectiveness of SA-dependent defenses, on the one hand, and JA/ET-dependent defenses, on the other hand, the range of effectiveness of ISR does not fully overlap with that of SAR (Ton *et al.*, 2002b). While the latter is operative mostly against biotrophic pathogens that are resisted through SA-dependent defenses, ISR also functions against necrotrophic pathogens that are susceptible to JA-responses, such as *A. brassicicola*.

Surprisingly, the transcriptional coactivator NPR1, necessary for the expression of SA-mediated defenses, was found to also play an essential role in ISR. In contrast to other mutations in the SA-signaling pathway, *npr1* plants do not express ISR upon root colonization by WCS417r (Pieterse *et al.*, 1998; Van Wees *et al.*, 2000). Because SAR is associated with NPR1-dependent *PR*-gene expression but 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 additively enhanced defensive capacity compared to that observed with either ISR or SAR alone (Van Wees *et al.*, 2000). These results suggest that the NPR1 protein is important in regulating and connecting different hormone-dependent induced defense pathways. However, to date the function of NPR1 in ISR has not been elucidated.

Whereas JA- and ET-dependent signaling pathways are required for the elicitation of WCS417r-ISR in *Arabidopsis*, alternative signaling pathways have been demonstrated in other plant-microbe combinations. (Van Loon & Bakker, 2005). For instance, in *Arabidopsis Bacillus* species elicit ISR independently of JA (Ryu *et al.*, 2004a), while *Serratia marcescens* mediates JA-dependent ISR against cucumber mosaic virus independently of NPR1 (Ryu *et al.*, 2004b). Although several rhizobacteria, including WCS417r and *S. marcescens*, are capable of producing SA under iron-limited conditions *in vitro*, induction of ISR is not abolished in NahG plants, indicating that SA is not involved (Van Loon & Bakker, 2005). Only in the case of *Pseudomonas aeruginosa* 7NSK2, induction of systemic resistance is abolished in NahG tobacco, though not in

NahG *Arabidopsis* (De Meyer *et al.*, 1999a). In *Arabidopsis*, the rhizobacterial strain *Paenibacillus alvei* K165 was shown to induce systemic resistance against *Verticillium dahliae*, which was blocked in the SA-signaling mutants *eds5* and *sid2* (Tjamos *et al.*, 2005), indicating that this rhizobacterial strain does activate an SA-dependent defense pathway.

WOUND-INDUCED RESISTANCE

To fend off insect herbivores, plants have adapted two distinct strategies: induced defense directed against the attacker (direct defense), and induced defense aimed at exploiting the natural enemies of the attacker (indirect defense). Both types of defense can be triggered upon insect feeding. Direct defense includes induced responses such as the production of secondary compounds or enzymes that act as toxins or feeding deterrents (Kessler & Baldwin, 2002; Howe, 2004), whereas indirect defense can involve production of extrafloral nectar (EFN) or a blend of volatiles that attracts predatory or parasitic enemies of the herbivorous insects (Heil & McKey, 2003; Turlings & Ton, 2006).

Direct defense

One of the best-studied examples of induced direct defense against herbivores is the rapid and systemic induction of proteinase inhibitors (PIs) after wounding or insect feeding in tomato (Howe, 2004). When a herbivore starts feeding on induced tissues, the PIs bind to, and inhibit, digestive proteases in the insect gut, blocking further consumption (Farmer & Ryan, 1992). Several PI-inducing signals have been identified, including oligogalacturonides (OGAs) and systemin. In response to wounding, OGAs are produced from cell-wall components, and the 18-amino acid peptide systemin is generated by cleavage from its precursor protein prosystemin. This eventually leads to JA synthesis through the octadecanoid pathway and induction of PIs and other defense-related proteins (Farmer & Ryan, 1992). The signal transduction events that couple the perception of OGAs and systemin at the plasma membrane to the subsequent activation of JA synthesis in the chloroplast remain to be elucidated (Howe, 2004).

The key role of JAs in induced direct defense against insect herbivores has been demonstrated in many plant-herbivore interactions. For instance, caterpillars of *P. rapae*, as well as green peach aphids (*Myzus persicae*) performed better on the *Arabidopsis* JA-signaling mutant *coi1* than on wild-type plants (Ellis *et al.*, 2002; Reymond *et al.*, 2004). Furthermore, the JA-biosynthesis mutant *fad3fad7fad8* is extremely sensitive to larvae of *B. impatiens* (McConn *et al.*, 1997). Similarly, the tomato JA-biosynthesis mutant *def1* (*defenseless 1*), has a compromised resistance to insect feeding (Howe *et al.*, 1996; Li *et al.*, 2002; Thaler *et al.*, 2002a).

Besides JA signaling, other hormones also influence plant resistance against insects. The ET-insensitive *Arabidopsis* mutant *ein2* is less resistant to larvae of the Egyptian cotton worm (*Spodoptera littoralis*) (Stotz *et al.*, 2000). In addition, *Arabidopsis* mutants and transgenics that are compromised in SA-dependent defense responses exhibit enhanced resistance against feeding by the cabbage looper *Trichoplusia ni* (Cui *et al.*, 2002). Thus, whereas JA plays a main role, ET and SA also modulate plant-insect interactions.

Indirect defense

Upon infestation by chewing insects, plants not only systemically activate the production of PIs and other direct defense mechanisms (Kessler & Baldwin, 2002; Howe, 2004), but also emit a complex bouquet of VOCs through which they attract natural enemies of the herbivorous insect (indirect induced defense; Turlings *et al.*, 1990; Van Poecke & Dicke, 2004; Rasmann *et al.*, 2005; Turlings & Ton, 2006). As is the case in the activation of direct defenses against insects, JA is the major signaling molecule involved in the induced production of plant volatiles (Van Poecke & Dicke, 2004). For instance, the volatiles induced upon feeding of *P. rapae* in the transgenic *Arabidopsis* S-12 line with reduced JA biosynthesis were less attractive to the parasitoid wasp *Cotesia rubecula* (Van Poecke & Dicke, 2002). Also ET and SA can influence the blend of VOCs that are produced upon infestation. ET was shown to enhance JA-mediated volatile emission in Lima bean (*Phaseolus lunatus*) (Horiuchi *et al.*, 2001). Herbivores such as spider mites induce emission of MeSA in many plant species (Ament *et al.*, 2004; De Boer & Dicke, 2004), which can lead to the activation of SA-inducible defense-related genes (Arimura *et al.*, 2000; Kant *et al.*, 2004). In line with these results, feeding by *P. rapae* larvae induced MeSA production in *Arabidopsis* (Van Poecke & Dicke, 2002). In NahG plants, MeSA was not produced, leading to a decreased attractiveness of the induced volatile blend to *C. rubecula* (Van Poecke & Dicke, 2002). These results clearly illustrate that JA, ET and SA all play a role in induced indirect defense against herbivorous insects.

Emission of VOCs not only attracts predatory or parasitoid enemies of the attacking herbivore, but can also enhance resistance of neighboring plants against subsequent insect attack (Baldwin *et al.*, 2006). However, in an evolutionary perspective, it has always been puzzling how this form of plant-plant communication can persist, as it benefits the receiver rather than the emitter plant. A possible explanation is the existence of plant-altruism. In a viscous plant population neighbors are likely to be genetic relatives. Under such circumstances, warning through VOC emission would positively affect hereditary transmission of genes that are closely related to those of the emitting individual. Analysis of a simulation model for emission of a secondary warning signal in a viscous plant population already indicated that altruism is likely to evolve when competition for nutrients and space is not too intense (Kobayashi & Yamamura, 2007).

VOCs might also play a role as systemic within-plant signaling compounds in insect-induced resistance. Indeed, recent findings by Heil & Silva Bueno (2007) and Frost *et al.* (2007) support a within-plant signaling role of VOCs. Controlled air flow from the emission space of infested leaves to that of distal plant parts resulted in a higher activation of defenses in exposed leaves of Lima bean (Heil & Silva Bueno, 2007) and an increased resistance to Asian gypsy moth (*Lymantria dispar*) infestation (Frost *et al.*, 2007), when compared to untreated distal plant parts. If this would be the primary function of wounding-induced VOCs, surrounding organisms, such as neighboring plants, herbivorous insects, and predatory or parasitoid insects may have merely evolved the ability to "eavesdrop" on this airborne within-plant signaling (Baldwin *et al.*, 2006; Heil & Silva Bueno, 2007).

CHEMICALLY INDUCED RESISTANCE

In addition to biological stimuli, the application of certain chemicals can also induce resistance in distal plants parts, to which they may be transported in the xylem. Often, these chemicals induce a similar resistance response as biologically induced SAR, as is the case upon application of synthetic SA, INA (2,6-dichloroisonicotinic acid) and BTH (benzothiadiazole) (Ward *et al.*, 1991; Uknes *et al.*, 1992; Lawton *et al.*, 1996). The non-protein amino acid ß-amino butyric acid (BABA) seems to induce a partially different induced resistance response. Application of BABA induces a resistance in many plant species (Jakab *et al.*, 2001; Cohen, 2002) that is effective against both biotrophic and necrotrophic pathogens (Zimmerli *et al.*, 2000; Ton & Mauch-Mani, 2004), insects (Hodge *et al.*, 2005), and certain abiotic stresses, such as osmotic and heat stress (Jakab *et al.*, 2005). This remarkably wide range of effectiveness of BABA-induced resistance (BABA-IR) suggests that multiple resistance responses are involved. Indeed, Zimmerli *et al.* (2000) demonstrated that BABA-IR against *H. parasitica* was still functional in *Arabidopsis* genotypes impaired in SA-dependent signaling, whereas BABA-IR against *P. syringae* pv. *tomato* was blocked in these genotypes. Hence, perception of BABA leads to the activation of multiple signal transduction pathways that all add to developing broad-spectrum BABA-IR.

By screening previously characterized *Arabidopsis* mutants for BABA-IR, ABA was identified as an additional regulator of BABA-IR against the necrotrophic fungi *A. brassicicola* and *Plectosphaerella cucumerina* (Ton & Mauch-Mani, 2004). Mutants impaired in SA, JA or ET signaling, or in camalexin production, all maintained BABA-IR against these fungi (Ton & Mauch-Mani, 2004). These findings suggested a novel role for ABA in the regulation of induced resistance against fungal pathogens. The role for ABA in BABA-IR was confirmed by the identification of the *ibs3* mutant (*impaired in BABA-induced sterility*). This mutant is affected in the transcriptional regulation of the ABA-biosynthetic gene *ABA1*, and concomitantly fails to express wild-type levels of BABA-IR against *H. parasitica* (Ton *et al.*, 2005).

PRIMING: DEFENSE MECHANISMS ON THEIR MARK

Induced resistance can be associated with the accumulation of defensive compounds such as PR-proteins with anti-microbial activity (Van Loon *et al.*, 2006b), PIs that reduce insect feeding (Howe, 2004), or volatiles that attract parasitoids and predators of the herbivores that feed on the plant (Van Poecke & Dicke, 2004). However, in many cases the enhanced defensive capacity in induced plants cannot be attributed to a direct activation of defense-related genes. Instead, the broad-spectrum protection of an induced plant is based on a faster and stronger activation of basal defense mechanisms upon subsequent exposure to microbial pathogens or herbivorous insects. Thus, 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 defense mechanisms is called 'priming' (Conrath *et al.*, 2002; 2006). Priming confers broad-spectrum protection without continuous expression of energy-costly defense mechanisms (Van Hulten *et al.*, 2006).

PRIMING DURING SAR

Indications for the involvement of priming of defense gene expression during SAR originally came from studies using elicitors of chemically induced resistance. In these studies, SA and BTH were applied at relatively low concentrations that did not activate defense responses directly, but rather primed the expression of *PAL* (*PHENYLALANINE AMMONIA-LYASE*) and *PR*-genes (Mur *et al.*, 1996; Kohler *et al.*, 2002). Mutant analyses demonstrated a role for NPR1 in the priming of SA-mediated defenses. Besides being blocked in direct activation of *PR*-genes, *npr1* plants were not able to prime expression of *PAL* for a faster response to virulent *P. syringae* pv. *tomato* when pre-treated with BTH or an avirulent strain of *P. syringae* pv. *tomato* (Conrath *et al.*, 2002; Kohler *et al.*, 2002). Furthermore, BTH-induced priming for enhanced deposition of callose-rich papillae upon infection by *H. parasitica* was also disrupted in *npr1*. Hence, NPR1 is required for priming of SA-mediated defense responses. In contrast, *edr1* (*enhanced disease resistance 1*) plants, which are mutated in a MAPKKK (Frye *et al.*, 2001), are constitutively primed for augmented expression of various SA-dependent defenses, such as *PR-1* expression and HR (Frye & Innes, 1998; Van Hulten *et al.*, 2006), suggesting that the EDR1 protein is a repressor of priming during SAR.

PRIMING DURING ISR

In contrast to SAR, WCS417r-ISR is not associated with direct induction or priming of *PR* gene expression (Van Wees *et al.*, 1999). To detect changes in gene expression upon elicitation of ISR in *Arabidopsis*, Verhagen *et al*. (2004) analysed the transcriptome of the

leaves upon colonization of the roots by WCS417r rhizobacteria. Although ISR was evident, no differences in gene expression were observed between the distal parts of ISR- and control-treated plants prior to pathogen challenge. However, a similar analysis after pathogen infection led to the identification of a set of genes that responded faster and stronger to pathogen attack (Verhagen *et al.*, 2004), in line with earlier observations on selected defense-related marker genes (Van Wees *et al.*, 1999). That primarily genes regulated by JA or ET showed this primed response upon pathogen attack confirmed earlier findings of priming of defense responses upon treatment with rhizobacteria. In *Arabidopsis*, pathogen-induced expression of the JA-responsive gene *VSP2*, as well as the production of ET, were augmented in WCS417r-pretreated plants compared to controls (Van Wees *et al.*, 1999; Hase *et al.*, 2003). The enrichment for particularly JA/ET-regulated genes among the primed ones, agrees with the requirement for JA- and ET- signaling in WCS417r-mediated ISR.

ISR-inducing *P. putida* LSW17S similarly primes JA/ET- and NPR1-dependent defense responses in *Arabidopsis* (Ahn *et al.*, 2007). Also in increased resistance induced by other plant growth-promoting rhizobacteria (PGPR) in various plant species, potentiated expression of defense-related genes has been observed (De Meyer *et al.*, 1999b; Ahn *et al.*, 2002; Kim *et al.*, 2004; Tjamos *et al.*, 2005). Other defense responses are similarly primed upon treatment with PGPR. Already in 1991, Van Peer *et al.* observed an increased accumulation of phytoalexins in stems of WCS417r-bacterized carnation upon inoculation with *Fusarium oxysporum* f. sp. *dianthi*, as compared to control-treated plants (Van Peer *et al.*, 1991). Pre-treatment of cucumber roots by biocontrol strains *S. marcescens* 90-166 and *P. fluorescens* 89B61 primed for accumulation of phenolic compounds upon challenge with *Colletotrichum orbiculare* (Jeun *et al.*, 2004). In pea, application of *Bacillus pumilus* SE34 enhanced cell-wall strengthening and barrier formation upon attack by the root-rotting fungus *Fusarium oxysporum* f. sp. *pisi* (Benhamou *et al.*, 1996). Systemic resistance induced by non-pathogenic fungi, such as *Trichoderma* spp. or mycorrhizal *Glomus mosseae* is also associated with priming of defense reactions (Cordier *et al.*, 1998; Pozo *et al.*, 2002; Shoresh *et al.*, 2005). Thus, the ability to prime systemic plant defense responses is widespread amongst ISR-triggering micro-organisms.

PRIMING DURING VOLATILE-INDUCED RESISTANCE

Priming by airborne signals, such as VOCs produced following insect herbivory, is a major topic in molecular-ecological research on plant–herbivore and plant–plant interactions (Baldwin *et al.*, 2006; Turlings & Ton, 2006). Analogous to chemicals such as INA and BTH, VOCs can either directly activate defense responses of recipient plants or prime them to respond faster and more strongly to stress exposure (Choh *et al.*, 2004; Engelberth *et al.*, 2004; Baldwin *et al.*, 2006; Turlings & Ton, 2006). In a laboratory study with maize, VOCs were demonstrated to prime neighboring plants for enhanced

direct and indirect defense, resulting in reduced performance of caterpillars of the Egyptian cotton leafworm *Spodoptera littoralis* and improved attractiveness to *Cotesia marginiventris* wasps, which feed on the insect (Ton *et al.*, 2007). Also in the field, herbivory-induced VOCs have been demonstrated to prime nearby plants for enhanced defense responses (Kessler *et al.*, 2006), indicating that priming for defense also occurs in nature. Recently, another demonstration of VOC-induced priming under natural conditions was provided by Heil & Silva Bueno (2007). They demonstrated that VOCs released by beetle-infested 'emitter' leaves of Lima bean plants growing in their natural habitat primed nearby 'receiver' leaves for enhanced secretion of extrafloral nectar, resulting in prolonged visitation by predatory arthropods. Though the active players in VOC-mediated priming differ amongst plant species, it seems to be a common defense strategy in plants (Baldwin *et al.*, 2006).

PRIMING DURING BABA-IR

Application of high concentrations of BABA directly activates defense responses that are regulated by SA or ABA (Van Hulten *et al.*, 2006; Van Hulten & Ton, unpublished results). However, lower amounts only prime the induction of these responses. In addition to increasing the induction of *PR-1* and other genes regulated by the SA pathway, BABA-IR against *H. parasitica* co-occurs with an enhanced formation of callose-containing papillae at entry sites of the pathogen (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). Together with putative antimicrobial components residing in the callose matrix, these papillae form a physical and/or chemical barrier for the pathogen, thereby preventing further invasion of the plant tissue. ABA-dependent enhancement of callose deposition is also involved in, and may even be essential for, BABA-IR against the fungal pathogens *A. brassicicola* and *P. cucumerina* (Ton & Mauch-Mani, 2004). ABA-deficient *aba1-5*, ABA-insensitive *abi4-1*, and callose-deficient *pmr4-1* were neither able to express BABA-IR, nor did they show BABA-induced augmentation of callose deposition at sites of pathogen penetration.

Application of high concentration of BABA renders *Arabidopsis* plants female-sterile (Jakab *et al.*, 2001). Screening for mutants that are impaired in BABA-induced sterility (*ibs*), resulted in the identification of three genes (*IBS1*, *IBS2/SAC1b* and *IBS3/ABA1*) with a regulatory role in BABA signaling (Ton *et al.*, 2005). In *ibs1*, *ibs2* and *ibs3*, BABA-IR was also impaired, whereas basal resistance was not. Bioassays in which diverse attackers were used for challenge, revealed that IBS1 is involved in the SA-dependent part of BABA-IR, while IBS2 and IBS3 are required for ABA-regulated callose deposition. Application of BABA to *ibs1* plants could still generate BABA-IR against necrotrophic fungi, but not against *P. syringae* pv. *tomato*. Conversely, BABA-IR in *ibs2* and *ibs3* mutant plants was still effective against the latter pathogen, but not against *A. brassicicola* and *P. cucumerina*.

MOLECULAR MECHANISMS OF PRIMING

Priming is a phenomenon that has been associated with different types of induced resistance (Conrath *et al.*, 2002; 2006), which boosts the inducible defenses that are activated in the host upon pathogen defense. Priming provides the plant with an enhanced capacity for rapid and effective activation of cellular defense responses when needed and allows it to react more effectively to any invader encountered. This defense mechanism can also explain the broad-spectrum effectiveness that is typical for many induced resistance phenomena. The molecular mechanisms underlying priming are still poorly understood. Hypothetically, the primed state is based on the accumulation, or post-translational modification of one or more signaling proteins that, after being expressed and/or modified, still remain inactive. Upon perception of a second pathogen-derived stress signal this enhanced defense signaling capacity would enable a faster and stronger defense reaction. TF proteins are likely candidates for being actors in this two-step regulatory mechanism. In this scenario, the pathogen-induced signal transduction in primed cells could directly induce a sufficient amount of defense-related genes, without the need of a preliminary step of TF expression.

TRANSCRIPTION FACTORS AND REGULATION OF DEFENSE

Arabidopsis is predicted to possess at least 1,572 (Riechmann, 2002) and potentially 2,077 TFs (Libault *et al.*, 2007). This roughly represents 6% of the total number of *Arabidopsis* genes. In the genomes of *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* this representation of transcriptional regulators is 1.3, 1.7 and 1.7 times lower, respectively (Riechmann *et al.*, 2000). In *Drosophila*, the large number TFs and their diversity have been suggested to be correlated to the substantial regulatory complexity of this organism (Adams *et al.*, 2000). As *Arabidopsis* contains relatively more TFs than *Drosophila*, this would imply that its regulation of the transcriptome is even more complex than that in the latter organism (Riechmann, 2002). Furthermore, based on the approximately 1,850 - 2,000 TFs per 30,000 - 40,000 genes of the human genome (Tupler *et al.*, 2001; Venter *et al.*, 2001), the transcriptional complexity of *Arabidopsis* seems to equal that of humans.

Out of the more than 45 different TF gene families, eight have been implicated in plant disease resistance (Jalali *et al.*, 2006). Genes encoding WRKY TFs are rapidly induced during defense reactions (Dong *et al.*, 2003; Li *et al.*, 2004). Moreover, a small number of NPR1-dependent WRKYs have been characterised as regulators of SAR (Wang *et al.*, 2006). Similarly, TGA TFs, which belong to the bZIP family, and *WHY1*, one of three *Arabidopsis* Whirly TFs (Desveaux *et al.*, 2005), have been demonstrated to be essential for SAR against *H. parasitica* (Zhang *et al.*, 2003; Desveaux *et al.*, 2004). AP2/ERFs are regulators of responses to various environmental cues, including abiotic

stresses (Fujimoto *et al.*, 2000; Park *et al.*, 2001) and pathogen infection (Solano *et al.*, 1998; Park *et al.*, 2001; McGrath *et al.*, 2005). Furthermore, two members of the AP2/ERF family, ERF1 (Lorenzo *et al.*, 2004) and ORA59 (Pré, 2006) are known to induce JA/ET-dependent pathogen defense genes and repress JA-regulate genes that are involved in the wounding response. Conversely, the bHLH (basic helix-loop-helix) TF MYC2, represses genes that are regulated by both JA and ET, while it induces expression of genes that are responsive to only JA (Boter *et al.*, 2004; Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). Of the more than 200 *Arabidopsis* MYB TFs, a small number is implicated in pathogen- or insect-induced defense signaling. MYB102 and MYB108 play a role in defense against insect herbivory and resistance against necrotrophic pathogens, respectively (Mengiste *et al.*, 2003; De Vos *et al.*, 2006a). Furthermore, MYB-like transcription factors have been demonstrated to function in hypersensitive cell death (MYB30; Raffaele *et al.*, 2006), production of defense-related glucosinolates (MYB34/ATR1; Celenza *et al.*, 2005), the wound response (MYB15; Cheong *et al.*, 2002) and regulation of the *PHENYLALANINE AMMONIA LYASE* (*PAL*) gene in response to wounding and elicitor treatment (Sugimoto *et al.*, 2000). Finally, some members of the DOF and NAC TF family have been reported to play a regulatory role in plant defense against microbial pathogens (Collinge & Boller, 2001; Yanagisawa, 2002). In conclusion, TFs are widely implicated in the regulation of diverse defense responses.

OUTLINE OF THIS THESIS

Use of the model plant *Arabidopsis* has resulted in several breakthroughs in the study of rhizobacteria-mediated ISR. The work described in this thesis aims to further identify key regulators in the signaling pathway controlling WCS417r-mediated ISR, ranging from early signaling steps upon local perception of the root-colonizing bacteria to the regulation of the onset of the primed defense state in leaf tissues.

Using Affymetrix 8000 GeneChips, Verhagen *et al.* (2004) demonstrated that colonization of the roots by WCS417r altered the local expression of about 100 genes. One of the WCS417r-responsive genes codes for the TF MYB72. Expression of the *MYB72* gene was up-regulated 2.8- and 3.1-fold in the roots at three and seven days after WCS417r application, respectively. *Chapter 2* focuses on the involvement of MYB72 in the early signaling events of the ISR pathway. Analysis of two knock-out mutants revealed that *MYB72* is indispensable for ISR against different pathogens. *Chapter 3* describes that MYB72 is also required for the systemic induction of resistance upon treatment of the roots with the non-pathogenic root-colonizing fungus *Trichoderma asperellum* T34. This fungal biocontrol agent failed to induce resistance in the *myb72-1* mutant against different types of pathogens and did not prime for enhanced expression of the JA-inducible *LOX2* gene.

Micro-array-based transcriptome analysis showed that treatment with WCS417r bacteria did not cause direct changes in gene expression in the leaves, but primed the pathogen-induced expression of mostly JA/ET-dependent genes (Verhagen *et al.*, 2004). Regulation of gene expression often occurs through binding of TFs to *cis*-acting elements in the promoter region. *Chapter 4* discusses the over-representation of a specific *cis*-acting element in the promoter regions of genes that are primed by *P. fluorescens* WCS417r for enhanced transcriptional induction upon treatment with MeJA or *P. syringae* pv. *tomato*. This *cis*-acting element serves as a docking site for the TF MYC2. Bioassays using mutants with a defect in the *MYC2* gene demonstrated that this transcription factor is essential for the onset of ISR.

Priming for augmented expression of defense-related genes has also been demonstrated to occur in other types of induced resistance, such as BABA-IR (Conrath *et al.*, 2006). *Chapter 5* describes a survey of the TFs involved in priming during ISR and BABA-IR. Furthermore, the chapter addresses differences and similarities between rhizobacteria-mediated ISR and BABA-IR.

Finally, in *Chapter 6* the results are discussed with reference to current ideas about induced disease resistance, and the role of priming.

CHAPTER 2

MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance

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ABSTRACT

Colonization of *Arabidopsis* roots by non-pathogenic *Pseudomonas fluorescens* WCS417r bacteria triggers a jasmonate- and ethylene-dependent induced systemic resistance (ISR) that is effective against a broad range of pathogens. Microarray analysis revealed that the R2R3-MYB-like transcription factor gene *MYB72* is specifically activated in the roots upon colonization by ISR-inducing WCS417r bacteria. Here we show that T-DNA knockout mutants *myb72-1* and *myb72-2* are incapable of mounting ISR against the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, the oomycete pathogen *Hyaloperonospora parasitica*, and the necrotrophic fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea*, indicating the MYB72 is essential to establish broad-spectrum ISR. Overexpression of *MYB72* in transgenic 35S::MYB72 plants did not result in enhanced resistance against any of the pathogens tested, demonstrating that MYB72 is not sufficient for the expression of ISR. Yeast two-hybrid analysis revealed that MYB72 can physically interact *in vitro* with the ETHYLENE INSENSITIVE3 (EIN3)-LIKE transcription factor EIL3, linking MYB72 function to the ethylene response pathway. However, WCS417r activated *MYB72* expression in ISR-deficient, ethylene-insensitive *ein2-1* plants. Moreover, exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) induced wild-type levels of resistance in *myb72-1*, suggesting that MYB72 acts upstream of ethylene in the ISR signaling pathway. Collectively, this study identified the transcriptional regulator MYB72 as a novel ISR signaling component that is required in the roots during early signaling steps of rhizobacteria-mediated ISR.

INTRODUCTION

The soil environment that is influenced by plant roots, the rhizosphere, is a nutrient-rich habitat providing niches for numerous micro-organisms. Amongst these, many fungi and bacteria with properties beneficial to plants are present (Marx, 2004; Pozo *et al.*, 2004). Some plant-beneficial bacteria, e.g. *Bacillus* and fluorescent *Pseudomonas* species (Kloepper *et al.*, 2004; Weller, 2007), have been reported to protect plants against pathogenic micro-organisms through different mechanisms, such as competition for nutrients, secretion of antibiotics, secretion of lytic enzymes and stimulation of the plant's defensive capacity (Bakker *et al.*, 2007). The latter phenomenon is commonly referred to as induced systemic resistance (ISR; Van Loon *et al.*, 1998). 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, viruses and even insect herbivores (Van Loon *et al.*, 1998; Van Loon & Bakker, 2005).

The ability of plants to develop ISR in response to root colonization by *Pseudomonas* bacteria depends on the host - rhizobacterium combination (Van Loon *et al.*, 1998; Pieterse *et al.*, 2002). The non-pathogenic, rhizobacterial strain *Pseudomonas fluorescens* WCS417r has been shown to trigger ISR in several plant species, and has served as a model strain to study ISR in *Arabidopsis* (Pieterse *et al.*, 2002). Colonization of *Arabidopsis* roots by WCS417r triggers ISR against the bacterial leaf pathogens *Xanthomonas campestris* pv. *armoraciae* and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), the fungal leaf pathogen *Alternaria brassicicola*, the oomycetous leaf pathogen *Hyaloperonospora parasitica* and the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997; Ton *et al.*, 2002b). Protection against these pathogens is characterized by a reduction in disease severity as well as an inhibition of pathogen growth.

Phenotypically, ISR resembles the systemic acquired resistance (SAR) that develops upon primary infection with a necrotizing pathogen (reviewed in Durrant & Dong, 2004). Although rhizobacteria-mediated ISR and pathogen-induced SAR are both effective against a broad spectrum of pathogens, their signal transduction pathways are distinct. The onset of SAR is accompanied by local and systemic increases in endogenous levels of salicylic acid (SA; Malamy *et al.*, 1990; Métraux *et al.*, 1990) and the transcriptional reprogramming of a large set of genes (Ward *et al.*, 1991; Maleck *et al.*, 2000), including genes encoding PATHOGENESIS-RELATED (PR) proteins (Van Loon *et al.*, 2006b). Some PR-proteins possess *in vitro* anti-microbial activity and are thought to contribute to the enhanced resistance state of SAR. Transduction of the SA signal requires functional NPR1 (NON-EXPRESSOR OF PR-PROTEINS 1), a regulatory protein that was identified in *Arabidopsis* through genetic screens for mutants impaired in their defense-response to SA or its functional analogs (Dong, 2004). Plants that carry a mutation in the *NPR1* gene accumulate normal or even higher levels of SA after pathogen infection, but

are impaired in their ability to transcriptionally activate *PR* genes and to mount a SAR response. Although some rhizobacterial strains can activate the SA-dependent SAR pathway (De Meyer & Höfte, 1997), the large majority of the reported resistance-inducing fluorescent *Pseudomonas* spp. strains have been shown to trigger ISR in a SA-independent manner (Van Loon & Bakker, 2005). WCS417r-mediated ISR functions independently of SA as well, as demonstrated by observations that *Arabidopsis* genotypes impaired in SA accumulation or biosynthesis (i.e. NahG, *eds5-1*, *sid2-2*) were still able to develop wild-type levels of ISR upon colonization of the roots by WCS417r (Pieterse *et al.*, 1996; 2002; Ton *et al.*, 2002a). Analysis of the jasmonic acid (JA)-response mutant *jar1-1*, a range of ethylene (ET)-response mutants, and the SAR-compromised mutant *npr1-1*, revealed that components of the JA- and the ET-response are required for triggering ISR and that this induced resistance response, like SAR, requires NPR1 (Pieterse *et al.*, 1998; Knoester *et al.*, 1999; Van Wees *et al.*, 2000). However, the ISR and the SAR signaling pathways diverge downstream of NPR1 because, unlike SAR, ISR is not marked by the transcriptional activation of PR genes (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997; Van Wees *et al.*, 1999).

In order to identify genes that mark the onset of ISR, the transcriptome of *Arabidopsis* was surveyed in roots and leaves upon colonization of the roots by ISR-inducing WCS417r bacteria (Verhagen *et al.*, 2004). Systemically in the leaves, no consistent changes in gene expression were observed in response to effective colonization of the roots by WCS417r, indicating that, in contrast to SAR, the onset of WCS417r-mediated ISR in the leaves is not associated with a major reprogramming of the transcriptome. However, after challenge inoculation of the induced plants with *Pst* DC3000, 81 genes showed a potentiated expression in the leaves, suggesting that these genes were primed to respond faster and/or more strongly upon pathogen attack. The majority of the primed genes appeared to be regulated by JA and/or ET signaling. Priming of pathogen-induced genes allows the plant to react more effectively to a subsequent invader, which might explain the broad-spectrum effectiveness of rhizobacteria-mediated ISR (Conrath *et al.*, 2002; 2006). In contrast to constitutive activation of defense responses, priming does not require major metabolic changes when no pathogens are present. Therefore, it forms a low-cost defense strategy while acting against a broad spectrum of attackers (Heil, 2002; Walters & Boyle, 2005; Van Hulten *et al.*, 2006).

Whereas in the leaves no changes in gene expression were evident before challenge inoculation, roots responded to colonization by ISR-inducing WCS417r bacteria with significant changes in the expression of 97 genes (Verhagen *et al.*, 2004). To investigate the biological role of the root-specific, WCS417r-inducible genes in the onset of ISR, we systematically started to analyze T-DNA insertion mutants of these genes. In this study, we demonstrate that the WCS417r-responsive gene *MYB72*, encoding a R2R3-MYB-like transcription factor protein, functions as an essential component during the early steps of the ISR signaling cascade in *Arabidopsis*. *MYB72* is a member of the large R2R3-MYB gene family of which 125 members have been identified in *Arabidopsis* (Kranz *et al.*, 1998; Stracke *et al.*, 2001; Yanhui *et al.*, 2006). R2R3-MYB transcription factors are

implicated in the regulation of various plant processes, although the function of most of them is still unknown (Stracke *et al.*, 2001).

RESULTS

Knockout mutant *myb72-1* is blocked in rhizobacteria-mediated ISR

Previously, microarray analysis revealed a large set of genes that showed an altered expression pattern in the roots upon colonization by ISR-inducing WCS417r rhizobacteria (Verhagen *et al.*, 2004). To investigate the role of these root-specific, WCS417r-induced genes in ISR signaling, we systematically analyzed knockout mutants of these genes for their ability to express WCS417r-mediated ISR against *Pst* DC3000. A mutant with a T-DNA insertion in the *MYB72* gene, which is specifically up-regulated in the roots upon colonization by WCS417r (Verhagen *et al.*, 2004; Fig. 2.1A), was identified as being ISR-non-responsive and was subjected to further detailed studies. Figure 2.1B shows that knockout mutant *myb72-1* (SAIL_713G10) was unable to mount ISR against Pst DC3000 in response to colonization of the roots by WCS417r.

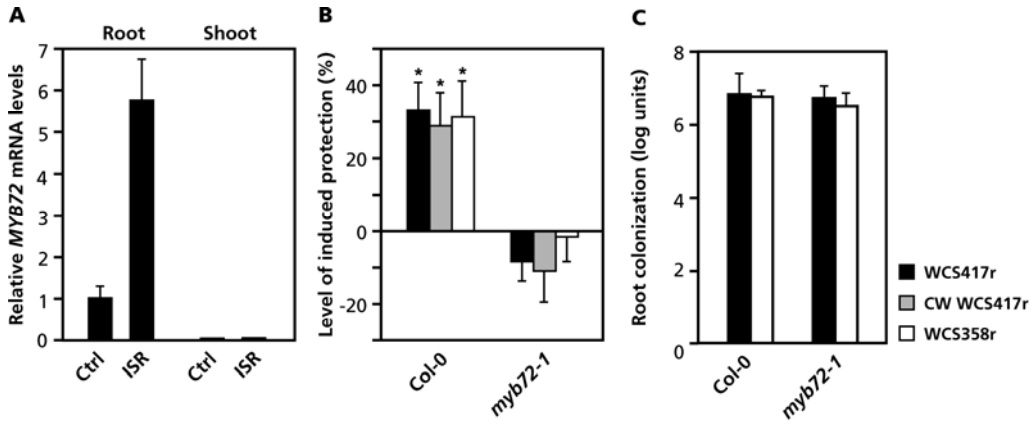

Figure 2.1. ISR against *Pst* DC3000 is blocked in *myb72-1*.

(A) **Q-PCR analysis of *MYB72* transcript levels in roots and shoots of Col-plants that were grown in soil for 2 weeks with or without ISR-inducing WCS417r bacteria.** (B) **Levels of induced protection against *Pst* DC3000 in Col-0 and knockout mutant *myb72-1*. ISR was induced by growing the plants for three weeks in soil containing living ISR-inducing WCS417r or WCS358r bacteria, or crude cell wall material of WCS417r (CW WCS417r). Five-week-old plants were challenge inoculated with a bacterial suspension of virulent *Pst* DC3000. Four days after challenge inoculation, the percentage of diseased leaves was assessed and the level of induced protection calculated on the basis of the reduction in disease symptoms relative to challenged, non-induced plants. Asterisks indicate statistically significant differences compared to non-induced control plants (Students *t*-test, α=0.05, n=20).** (C) **Numbers of rifampicin-resistant WCS417r or WCS358r bacteria (log^{10} of the number of colony forming units (cfu).ml^{-1}) in the rhizosphere of the plants at the end of the bioassay. In the rhizosphere of non-induced plants, no rifampicin-resistant bacteria were detected (detection limit 10^3 cfu.g^{-1} root fresh weight (FW)).**

Previously, rhizobacterial strain *Pseudomonas putida* WCS358r and a crude cell-wall preparation of WCS417r were demonstrated to trigger the ISR signaling pathway in *Arabidopsis*, resulting in a similar level of induced protection against *Pst* DC3000 as ISR induced by live WCS417r bacteria (Van Wees *et al.*, 1997). To find out whether ISR triggered by these inducers is also blocked in *myb72-1*, roots of Col-0 and *myb72-1* plants were treated with killed WCS417r cells, or with living WCS358r bacteria, and tested for the expression of ISR. Col-0 plants treated with crude WCS417r cell wall material or living WCS358r bacteria both showed comparable levels of protection against *Pst* DC3000 to that induced by live WCS417r cells (Fig. 2.1B). Knockout mutant *myb72-1* was unable to mount ISR in response to any of the inducers, confirming that MYB72 is required for the onset of ISR.

To investigate whether the impaired ISR response of *myb72-1* was caused by insufficient root colonization by the rhizobacterial strains, the number of rifampicin-resistant WCS417r and WCS358r bacteria per gram of root fresh weight was determined. No differences in the extent of root colonization between Col 0 and *myb72-1* plants were observed (Fig. 2.1C). Thus, the inability of *myb72-1* to express WCS417r-mediated ISR was not caused by reduced root colonization.

To confirm that the ISR-minus phenotype of knockout mutant *myb72-1* was caused by disruption of the *MYB72* gene, a second, independent T-DNA insertion mutant, designated *myb72-2* (SALK_052993), was tested for its ability to express WCS417r-mediated ISR. Figure 2.2 shows that *myb72-2*, as *myb72-1*, was unable to mount ISR against *Pst* DC3000, indicating that a functional MYB72 protein is required for the onset of WCS417r-mediated ISR against this pathogen in *Arabidopsis*.

Verification of T-DNA insertion sites in *myb72-1* and *myb72-2*

To verify the predicted T-DNA insertion sites of the *myb72* knockout mutants (Fig. 2.3A), genomic DNA flanking the T-DNA insertion was amplified using the PCR procedure described by Sessions *et al.* (2002). Amplification of a PCR product from genomic DNA

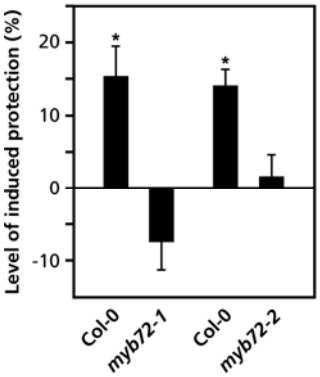

Figure 2.2. ISR against *Pst* DC3000 is blocked in *myb72* knockout mutants.

Quantification of WCS417r-induced protection against *Pst* DC3000 in wild-type Col-0 and knock-out mutants *myb72-1* and *myb72-2*. The level of induced protection was calculated as described for Figure 2.1. Asterisks indicate statistically significant differences compared to non-induced control plants (Students *t*-test, α=0.05, n=20). Bioassays were repeated with similar results. Error bars represent standard errors.

of Col-0 with *MYB72*-specific forward (F) and reverse (R) primers (Supplementary Table 2.1) that were predicted to anneal left and right of the T-DNA insertions, yielded PCR-products only when genomic DNA of Col-0 was used as a template (Fig. 2.3B). Amplification on genomic DNA of the *myb72* mutants did not result in a PCR product, suggesting that the *MYB72* gene was indeed disrupted by the large T-DNA insertions, resulting in a fragment that is too large to be amplified under the PCR conditions used. PCR analysis using a T-DNA left border primer in combination with the appropriate *MYB72*-specific primer, resulted in a PCR product in the *myb72* knockouts only. Sequence analysis of these amplicons confirmed the presence of a T-DNA insertion 51 bp and 736 bp downstream of the MYB72 translation start codon in *myb72-1* and *myb72-2*, respectively (data not shown). Since both mutants were impaired in their ability to mount WCS417r-mediated ISR against *Pst* DC3000, mutant *myb72-1* was used for further experiments.

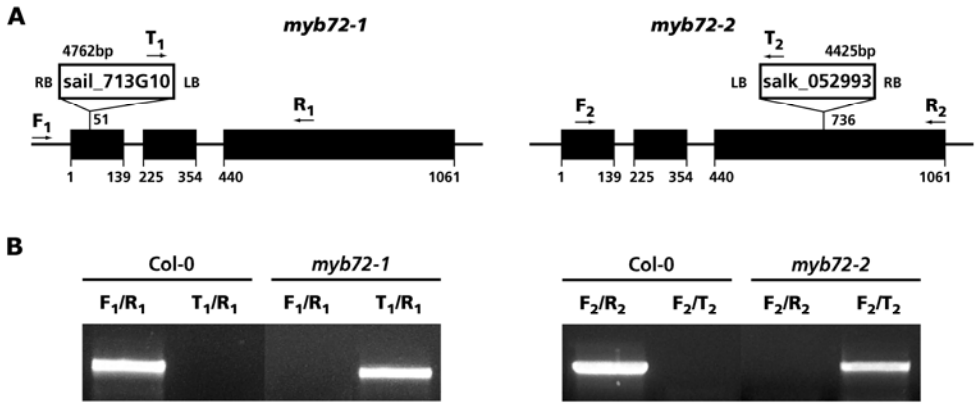

Figure 2.3. Verification of T-DNA insertion sites in *myb72* mutants.

(A) **Structure of the *MYB72* gene and position of the T-DNA insertions in *myb72-1* and *myb72-2* mutants. Exons are indicated as black boxes. The nucleotide numbers underneath indicate the start and the end of the exons. The 4762-bp T-DNA insertion in *myb72-1* is located 51 bp downstream of the predicted start codon of the *MYB72* open reading frame. In *myb72-2*, the 4425-bp T-DNA insert is located in the third exon, 736 bp from the start codon. The primers used for verification of the position of the T-DNA insertions are indicated by arrows. LB, left border of the T-DNA; RB, right border of the T-DNA; F_1 and F_2, *MYB72* forward primers; R_1 and R_2, *MYB72* reverse primers; T_1 and T_2, T-DNA left border primers.** (B) **PCR amplification using T-DNA left border, *MYB72* forward, and *MYB72* reverse primers, as indicated, on genomic DNA of Col-0, *myb72-1* and *myb72-2* plants, respectively.**

myb72-1 is not impaired in SAR or resistance induced by MeJA or ACC

To investigate the effect of the *myb72-1* mutation on pathogen-induced SAR, we compared the levels of rhizobacteria-mediated ISR and pathogen-induced SAR in this mutant. SAR was induced three days prior to challenge inoculation with virulent *Pst* DC3000 by infiltrating three lower leaves with avirulent *Pst* DC3000 (*avrRpt2*). Wild-

type Col-0 plants developed significant levels of protection against *Pst* DC3000 in response to induction of ISR and SAR (Fig. 2.4A). In contrast to ISR, SAR was expressed to wild-type levels in *myb72-1*, indicating that the ability to develop SAR was not altered in this mutant. Similarly, chemical induction of SAR by exogenous application of SA resulted in similar levels of protection against *Pst* DC3000 in Col-0 and *myb72-1* (Fig. 2.4B), confirming that *myb72-1* is not impaired in SAR.

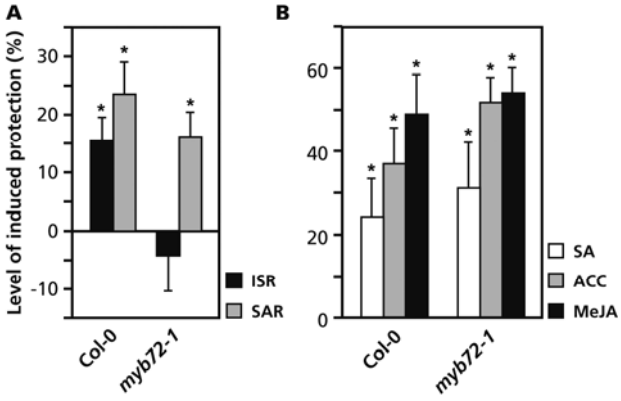

Figure 2.4. ISR, SAR, and chemically-induced resistance against *Pst* DC3000 in Col-0 and *myb72-1*.

(A) **Quantification of the level of ISR and SAR against *Pst* DC3000 in Col-0 and *myb72-1* plants. ISR was induced by treatment of the roots with WCS417r bacteria. Induction of SAR was performed three days before challenge inoculation by pressure infiltrating three lower leaves with a suspension of avirulent *Pst* DC3000(*avrRpt2*) bacteria.** (B) **Level of induced resistance against *Pst* DC3000 in Col-0 and *myb72-1* after exogenous application of either SA, ACC, or MeJA. Chemical inductions were performed by applying 1 mM SA, 1 mM of ACC, or 0.1 mM MeJA, as a soil drench seven and four days prior to challenge inoculation with *Pst* DC3000. For details on *Pst* DC3000 bioassays see legend to Figure 2.1.**

Like rhizobacteria-mediated ISR, exogenous application of methyl JA (MeJA) or the ET precursor 1-aminocyclopropane-1-carboxylate (ACC) triggers an enhanced level of resistance against *Pst* DC3000 (Van Wees *et al.*, 1999). To examine the effect of the *myb72-1* mutation on resistance induced by these chemicals, Col-0 plants were pretreated with MeJA or ACC at seven and four days before challenge inoculation with virulent *Pst* DC3000. Figure 2.4B shows that *myb72-1* developed wild-type levels of protection against *Pst* DC3000 in response to both chemicals, indicating the *myb72-1* mutation has no effect on the ability to express enhanced resistance in response to ACC or MeJA. These findings suggest that MYB72 operates upstream of JA and ET in the ISR signaling pathway.

MYB72 is required for ISR against a broad spectrum of pathogens

WCS417r-mediated ISR is effective against a broad spectrum of pathogens (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997; Ton *et al.*, 2002b). To examine whether MYB72 is required for the onset of broad-spectrum ISR, we tested the ability of *myb72-1* to express ISR against the biotrophic oomycete *H. parasitica* and the necrotrophic fungi *A. brassicicola* and *B. cinerea*. Figure 2.5A shows that WCS417r-mediated ISR and BTH (benzothiadiazole)–induced SAR resulted in a relatively moderate, but statistically significant, level of protection of Col-0 plants against *H. parasitica*. In similarity to the bioassays with *Pst* DC3000, mutant *myb72-1* plants failed to develop ISR against this pathogen, whereas induction of SAR resulted in wild-type levels of induced resistance. To test the effectiveness of ISR against *A. brassicicola*, ISR bioassays were performed in

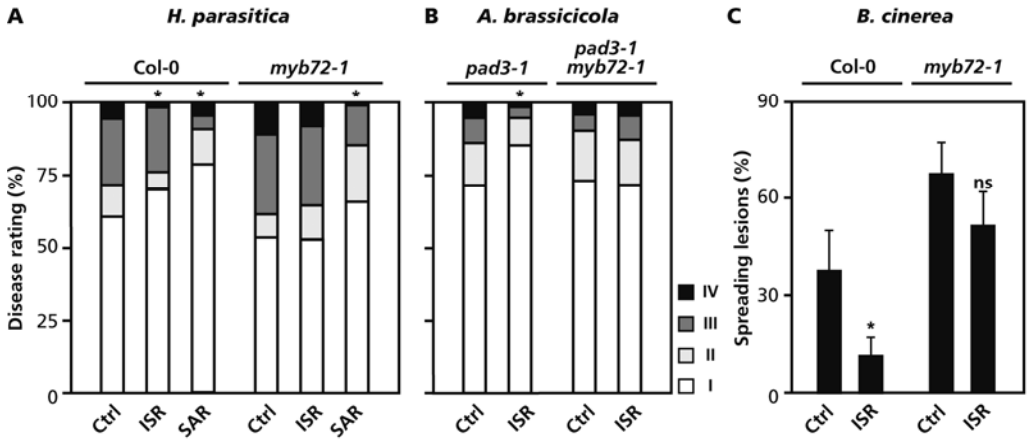

Figure 2.5. ISR against *H. parasitica*, *A. brassicicola*, and *B. cinerea* is blocked in *myb72-1*.

(A) **Quantification of ISR and SAR against *H. parasitica*. ISR was induced by growing the plants in soil containing ISR-inducing WCS417r bacteria. SAR was induced by applying 300 µM BTH as a soil drench three days before challenge. Plants were challenge inoculated with *H. parasitica* when three weeks old. Disease severity was determined nine days after challenge. Disease ratings are expressed as the percentage of leaves (n = ~250) in disease-severity classes: I, (no sporulation); II, (trailing necrosis); III, (< 50% of the leaf area covered with sporangia); IV, (> 50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse). Asterisks indicate statistically significantly different distributions of the disease severity classes compared with the non-induced control treatments (Chi-square, α=0.05).** (B) **Quantification of ISR against *A. brassicicola* in *pad3-1* and *pad3-1*/*myb72-1*. ISR was induced as described above. Plants were inoculated with *A. brassicicola* when five weeks old. Disease symptoms were determined five days after challenge. Disease ratings are expressed on the basis of symptom severity: I, no visible disease symptoms; II, non-spreading lesion; III, spreading lesion without tissue maceration; IV, spreading lesion with tissue maceration and sporulation of the pathogen. Asterisks indicate statistically significantly different distributions of the disease severity classes compared with the non-induced control treatments (Chi-square, α=0.05, n =120).** (C) **Quantification of ISR against *B. cinerea* in Col-0 and *myb72-1*. ISR was induced as described above. Plants were inoculated with *B. cinerea* when five weeks old. Disease symptoms were determined five days after challenge. Disease ratings were expressed as percentage of leaves showing spreading lesions. Asterisks indicate statistically significant differences compared to non-induced control plants (Students *t*-test, α=0.05, n=20). ns is abbreviation for non-significant. Error bars represents standard errors. All experiments were repeated at least once with similar results.**

the genetic background of the susceptible camalexin-deficient mutant *pad3-1*. Figure 2.5B shows that induction of ISR in *pad3-1* significantly reduced disease symptoms caused by *A. brassicicola* infection, whereas the *pad3-1/myb72-1* double mutant failed to mount ISR against this pathogen. Similarly, Col-0 plants, but not *myb72-1*, expressed statistically significant levels of ISR against *B. cinerea* (Fig. 2.5C). Together, these results demonstrate that MYB72 is essential for ISR against a broad spectrum of pathogens.

WCS417r-induced priming for enhanced callose deposition is blocked in *myb72-1*

Induced resistance against *H. parasitica* is associated with enhanced deposition of callose-containing papillae at sites of attempted penetration (Kohler *et al.*, 2002; Ton *et al.*, 2005). Figure 2.6 shows that induction of ISR by WCS417r, or SAR by exogenous application of BTH, resulted in a significantly decreased efficiency of *H. parasitica* spores to penetrate due to the enhanced formation of callose-containing papillae around the entry sites. Treated mutant *npr1-1* plants that are blocked in their ability to express both ISR and SAR, did not show this potentiated deposition of callose at the sites of spore penetration, confirming that priming for enhanced callose deposition is associated with ISR and SAR. Priming for enhanced callose deposition was normally expressed in *myb72-1* upon induction of SAR with BTH, but was absent in *myb72-1* plants of which the roots were treated with ISR-inducing WCS417r bacteria. Hence, priming for enhanced formation of callose-containing papillae is not impaired in *myb72-1*, but not expressed when triggered by ISR-inducing WCS417r bacteria. Thus, MYB72 plays an important role in the onset of this primed defense response during ISR.

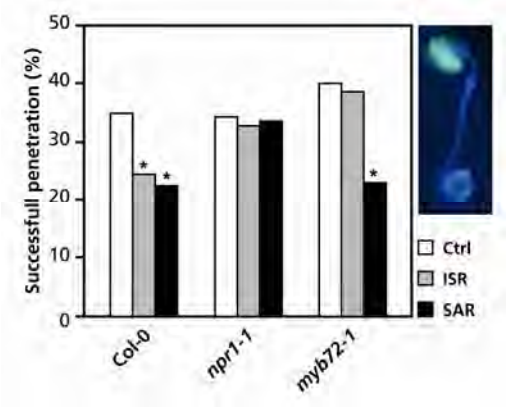

Figure 2.6. Mutant *myb72-1* is impaired in WCS417r-mediated priming for enhanced callose deposition at *H. parasitica* infection sites.

Induced resistance against *H. parasitica* is associated with enhanced deposition of callose-containing papillae at sites of attempted penetration (inset), resulting in a reduction of the number of spores that successfully penetrate into *Arabidopsis* leaves. Two days after challenge with *H. parasitica*, successful penetration was quantified in leaves of Col-0, *npr1-1* and *myb72-1* plants. Leaves of plants of which the roots were pre-treated with water (Ctrl), WCS417r (ISR) or BTH (SAR) were stained with Calcofluor/aniline blue and analyzed by epifluorescence microscopy (UV) and the percentage of germinating spores that did not lead to callose-deposition in the epidermal cell layer was determined (successful penetration). Inset shows a representative example of germinating *H. parasitica* spore triggering callose deposition in the underlying epidermal cell.

MYB72 functions upstream of or in parallel with ET signaling in the ISR pathway

MYB72 transcripts accumulate in the roots upon colonization by WCS417r, whereas systemically in the leaves, they are not detectable (Verhagen *et al.*, 2004; Fig. 2.1A). Hence, MYB72 is likely to play a role in the early steps of the ISR signaling pathway. Previously, Knoester *et al.* (1999) demonstrated that for the onset of ISR, ET signaling is required at the site of application of the ISR inducer. To investigate whether *MYB72* gene expression is regulated by ET, *MYB72* transcript levels were monitored in the roots by Q-PCR upon application of 0.1 mM ACC. Figure 2.7A shows that the ET-responsive gene *EBF2* (*EIN3 BINDING FACTOR 2*) (Guo & Ecker, 2003) is activated in ACC-treated roots. By contrast, *MYB72* is not activated upon ACC treatment, indicating that *MYB72* gene expression is not regulated by ET.

To test whether WCS417r-induced expression of *MYB72* in the roots requires ET sensitivity, *MYB72* transcript accumulation was examined in the ET-insensitive, ISR-minus mutant *ein2-1*. Colonization of the roots by WCS417r bacteria activated *MYB72* equally in both Col-0 and *ein2-1* (Fig. 2.7B). These results indicate that MYB72 either acts upstream of ET signaling, or is co-required with components from the ET signaling pathway during the onset of ISR.

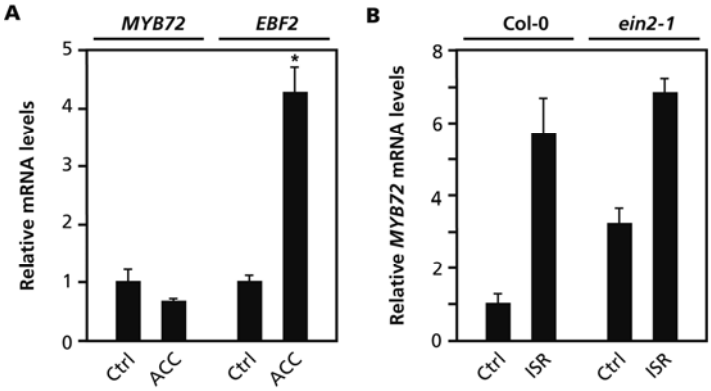

Figure 2.7. MYB72 expression is not regulated by ET.

(A) **Q-PCR analysis of *MYB72* transcript levels in the roots of five-week-old Col-0 plants of which the roots were treated with a soil drench of water or 0.1 mM ACC. To check the effectiveness of the ACC treatment, expression levels of the ET-responsive *EBF2* gene were checked in the same samples.** (B) **Q-PCR analysis of *MYB72* transcript levels in the roots of Col-0 and *ein2-1* plants, two weeks after transfer of the seedlings to soil containing WCS417r bacteria or not. mRNA levels in water-treated control plants were set at 1.**

MYB72 is required but not sufficient for ISR

To investigate whether MYB72 is not only required but also sufficient for the onset of ISR, transgenic plants that constitutively express *MYB72* (35S::MYB72) were generated

and tested for enhanced disease resistance. Seven independent homozygous T3 lines (OX1 to OX7) were phenotypically characterized. All transgenic lines displayed a phenotype that was similar to the parental Col-0 line and the empty vector (EV) control (Supplementary Figure 2.1). RNA blot analysis of roots and shoots of the 35S::MYB72 lines confirmed constitutive expression of *MYB72* in all lines, be it to varying levels (Fig. 2.8A). Bioassays for induced resistance assays were performed with Col-0, the EV control and the seven 35S::MYB72 transgenic lines. Figure 2.8B shows that the level of resistance against *Pst* DC3000, *H. parasitica*, and *B. cinerea* in lines OX2 and OX7 was not significantly enhanced compared to from that in the EV control line. Resistance assays with the other OX-lines yielded similar results (data not shown), indicating that ectopic expression of *MYB72* is not sufficient for the onset of ISR.

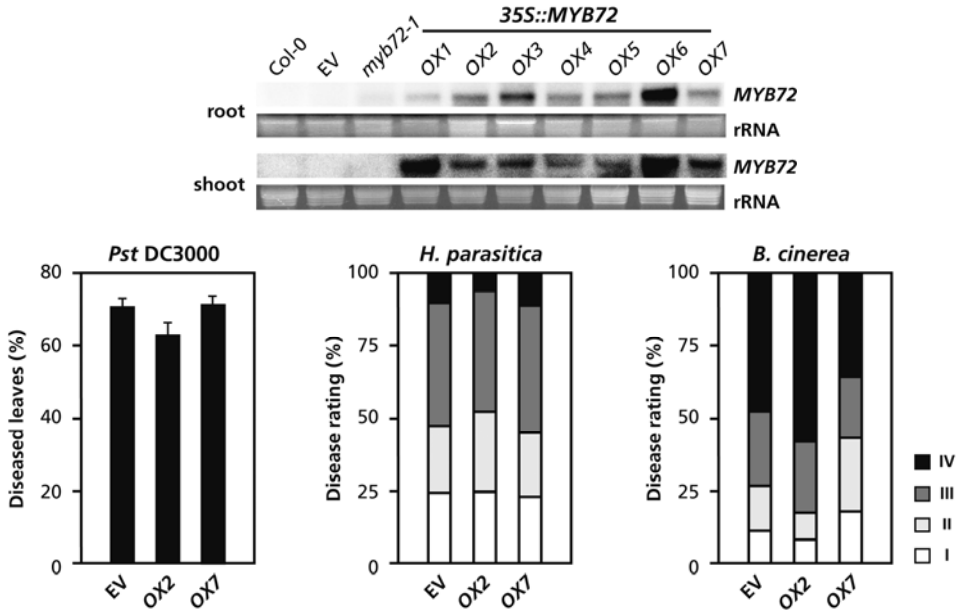

Figure 2.8. 35S::MYB72 overexpressors do not show enhanced levels of disease resistance.

(A) **Northern blot analysis of *MYB72* transcript levels in roots of five-week-old wild-type Col-0 plants and the mutant and transgenic lines EV (empty vector control), *myb72-1*, and 35S::MYB72-OX1 to OX7. To check for equal loading, rRNA bands were stained with ethidium bromide.** (B) **Levels of disease severity in EV, OX2 and OX7 upon inoculation with *Pst* DC3000, *H. parasitica*, and *B. cinerea*. 35S::MYB72 overexpressing lines OX1, OX3, OX4, OX5 and OX6 displayed similar levels of disease severity as OX2 and OX7 (not shown). For details on pathogen bioassays see legends to Figures 2.1 and 2.3.**

MYB72 physically interacts with EIL3 *in vitro*

If MYB72 is essential but not sufficient for the onset of ISR, then additional components are likely to be co-required. Transcription factors usually exert their action in a complex

with other proteins. Earlier, a systematic search for proteins that physically interact with MYB transcription factors was initiated by members of the EU-funded REGIA (Regulatory Gene Initiative in *Arabidopsis*) consortium. Using the ProQuest yeast two-hybrid system (Invitrogen), this screen revealed that MYB72 physically interacts with the ETHYLENE INSENSITIVE 3 (EIN3)-like protein EIL3 (At1g73730; data not shown). To confirm the interaction of MYB72 with EIL3, the full-length coding regions of *MYB72* and *EIL3* were isolated, fused to the DNA binding domain (BD) and transcription activation domain (AD) of GAL4, and tested in a yeast two-hybrid assay. Figure 2.9A shows that cells containing the BD::MYB72 fusion with the AD::EIL3 fusion, and cells containing both the BD::EIL3 and the AD::MYB72 fusion were capable of growth on selective dropout (SD) medium, to which 100 mM of the histidine biosynthesis inhibitor 3-amino-1,2,4-triazole (3AT) was added. Cells containing either AD::MYB72 or AD::EIL3 in combination with GAL4 BD fused to the full-length *Arabidopsis* FRIGIDA protein (BD::FRI; Rutjens, 2007), which were used as negative controls, did not grow on the selective medium. Cells containing either the BD::MYB72 or the BD::EIL3 fusion with the empty GAL4 AD vector showed a low level of growth, indicating a low level of auto activation of the reporter gene in these combinations. Together these results indicate that MYB72 and EIL3 interact in vitro.

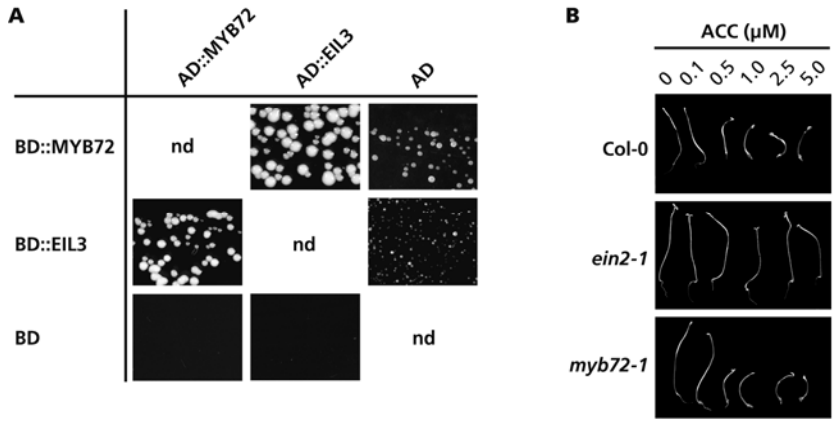

Figure 2.9. MYB72 interacts with EIL3 in a yeast two-hybrid assay.

(A) **Yeast two-hybrid assay of interactions between the transcription factors MYB72 and EIL3 fused to the GAL4 DNA-binding domain (BD) or transcriptional-activation (AD) domain. Yeast cells (PJ69-4A) containing either DB::MYB72 and AD::EIL3, or DB::EIL3 and AD::MYB72, or either of these fusions together with the vector control were grown on yeast selective dropout (SD) medium. To suppress histidine formation that results from autoactivated HIS3-reporter gene activity, 100 mM of the histidine biosynthesis inhibitor 3AT was added to the medium and yeast cells were grown for three days at 20°C.** (B) **Triple response assay of etiolated Col-0, *ein2-1* and *myb72-1* seedlings grown for seven days in the dark at 20°C on MS agar containing 0, 0.1, 0.5, 1.0, 2.5 or 5.0 μM ACC.**

The EIL3 paralogs EIN3, EIL1 and EIL2 have been demonstrated to function as key transcription factors of ET-regulated gene expression and to act as positive regulators of ET signaling (Stepanova & Ecker, 2000). Since MYB72 acts upstream of ET signaling or is co-required with components of the ET signaling pathway during the onset of ISR (Fig. 2.5), we investigated whether the ISR-minus phenotype of the *myb72-1* knockout mutant is caused by a reduced sensitivity to ET. The "triple response" is a reaction of etiolated seedlings to ET, and is commonly used as a reliable marker for ET sensitivity (Guzmán & Ecker, 1990). Etiolated Col-0, ET-insensitive *ein2-1* and *myb72-1* seedlings were grown in the dark on MS-agar plates with or without ACC. Ten days after germination, Col-0 seedlings grown on a concentration range of ACC showed a typical ET-induced growth inhibition of the hypocotyl and root, both characteristics of the triple response (Fig. 2.9B). As expected, the triple response was not apparent in the ET-insensitive *ein2-1* seedlings. In contrast, in mutant *myb72-1* seedlings, the triple response was indistinguishable from that in wild-type Col-0 plants. These results demonstrate that the absence of a functional MYB72 protein does not affect ET sensitivity. Hence, the inability of *myb72-1* plants to mount ISR is not caused by an inability to react to ET in this mutant.

DISCUSSION

Colonization of the roots of *Arabidopsis* by non-pathogenic fluorescent *Pseudomonas* bacteria, such as WCS417r and WCS358r, leads to an enhanced level of resistance against a broad spectrum of pathogens in foliar tissues (Pieterse *et al.*, 2002). Genes of which the expression is changed in the roots upon colonization by ISR-inducing rhizobacteria are potentially involved in the onset of ISR. In a previous study, we identified 97 *Arabidopsis* genes that show this characteristic (Verhagen *et al.*, 2004). Here, we demonstrate the role of one of these genes, *MYB72*, in the onset of rhizobacteria-mediated ISR. The transcription factor gene *MYB72* is specifically expressed in the roots upon colonization by ISR-inducing WCS417r bacteria. T-DNA insertions in the *MYB72* gene resulted in the inability to express rhizobacteria-mediated ISR against different types of pathogens (Fig. 2.1, 2.2, 2.5, and 2.6). Pathogen- or chemically induced SAR was not affected in *myb72* mutant plants (Figs. 2.4 and 2.5A), indicating that the mutations in *MYB72* specifically affect rhizobacteria-induced resistance. These findings indicate that MYB72 plays a role in the onset of ISR in the roots and is required for systemic activation of broad-spectrum ISR in the leaves.

MYB72 is part of a transcription factor family of which several members are involved in stress signaling

MYB72 is a member of a large class of genes that contain one or more *MYB* domains (Stracke *et al.*, 2001). *MYB* genes were first identified as oncogenes derived from

retroviruses in animal cells (Klempnauer *et al.*, 1982). They encode transcription factor proteins that share the conserved *MYB* DNA-binding domain (Jin & Martin, 1999). MYB proteins are categorized into subfamilies depending on the number of conserved repeats of the MYB domain. The ones from animals generally contain three MYB repeats, which are referred to as R1, R2 and R3. Most of the *MYB*-like genes in plants have only the R2 and R3 repeats (Kranz *et al.*, 1998). According to an inventory of the *Arabidopsis* genome, *MYB72* is one of approximately 125 genes that encodes a putative R2R3-MYB protein in this plant species (Stracke *et al.*, 2001). Gene expression analyses suggest a role for many R2R3-MYB proteins in a range of activities, such as plant secondary metabolism, development, regulation of cell death, stress tolerance, and pathogen resistance (Stracke *et al.*, 2001; Yanhui *et al.*, 2006). However, the biological functions of most of the MYB-like transcription factors have not been determined.

Within *Arabidopsis*, the MYB72 protein was found to possess highest homology with MYB10, MYB58, and MYB63 (Stracke *et al.*, 2001), of which the functions are currently unknown. Alignment of the R2R3 domain of MYB72 with amino acid sequences of other plant species in the databases further revealed high homology with the MYB-like transcription factor protein OsLTR1 from rice (75% identity; GeneBank accession AAP92750), which has been implicated in JA-dependent defense responses (NCBI database locus information), and ZmMRP1 from maize (75% identity; GeneBank accession S04898) (Supplementary Figure 2.2). Besides MYB72, several other *Arabidopsis* MYB-like transcription factors have also been implicated to function in biotic or abiotic stress signaling. Mengiste *et al.* (2003) identified BOS1 (MYB108) and demonstrated a role for this protein in resistance against necrotrophic pathogens. Outside the conserved R2R3 domain, the amino acid sequence of MYB72 has no significant homology with that of BOS1, suggesting that both MYB transcription factors are not functionally related. This is confirmed by our observation that, in contrast to *BOS1*, overexpression of *MYB72* does not result in enhanced resistance to necrotrophic pathogens (Figure 2.6B). Other *Arabidopsis* MYB-like transcription factors have been demonstrated to function in drought stress responses (MYB2; Abe *et al.*, 2003), hypersensitive cell death (MYB30; Raffaele *et al.*, 2006), production of defense-related glucosinolates (MYB34/ATR1; Celenza *et al.*, 2005), the wound response (MYB15; Cheong *et al.*, 2002), and defense against insect herbivory (MYB102; De Vos *et al.*, 2006a). Also in other plants species MYB-like transcription factors play a role in the regulation of stress responses. For example, tobacco NtMYB2 was shown to positively regulate the expression of the *PHENYLALANINE AMMONIA LYASE* (*PAL*) gene in response to wounding and elicitor treatment (Sugimoto *et al.*, 2000), whereas rice OsMYB4 was shown to function as a key regulator in cold tolerance (Vannini *et al.*, 2004).

Specificity of *MYB72* gene expression

Previously, Kranz *et al.* (1998) analyzed the expression patterns of a large set of *Arabidopsis* MYB genes in different plant organs and under various conditions, such as

treatment with various hormones, exposure to abiotic stress, and infection by *Pst* DC3000. In this study *MYB72* transcripts were not detected in any of the organs or conditions tested. Analysis of the *Arabidopsis* transcriptome using the *Arabidopsis* microarray database and analysis toolbox Genevestigator (Zimmermann *et al.*, 2004), confirmed that *MYB72* is not activated in response to any of the hormones or biological agents tested (data not shown), suggesting that the induction of *MYB72* in the roots upon colonization by non-pathogenic rhizobacteria is highly specific. However, low iron conditions or treatment of *Arabidopsis* roots with an excess amount of zinc do induce the expression of *MYB72* in the roots (Colangelo & Guerinot, 2004; Van de Mortel *et al.*, 2006). Earlier studies by Thomine *et al.* (2003) and Van de Mortel *et al.* (Van de Mortel *et al.*, 2006) demonstrated that high zinc availability distorts iron uptake by the plant, thereby mimicking the iron-limiting conditions that activate *MYB72*. Interestingly, fluorescent *Pseudomonas* spp., such as WCS417r and WCS358r, produce large quantities of iron-chelating siderophores that facilitate the uptake of iron by the bacteria, thereby depriving their direct vicinity from iron (Bakker *et al.*, 2007). Hence, it is not inconceivable that the induction of *MYB72* is caused by enhanced iron stress that is inflicted in the roots upon colonization by the ISR-inducing rhizobacteria. This can be supported by the fact that both iron deprivation (Connolly *et al.*, 2003) and bacterization by WCS417r (Verhagen *et al.*, 2004) lead to induction of the expression of *FERRIC REDUCTION OXIDASE* 2 (*FRO2*) (Robinson *et al.*, 1999), a gene encoding a protein that plays a crucial role in the uptake of iron by the roots.

MYB72 is required in early ISR signaling

Previously, Knoester *et al.* (1999) demonstrated that ET signaling is required in the roots for the expression of ISR in the leaves. It was shown that mutant *eir1-1*, which is insensitive to ET in the roots only (Roman *et al.*, 1995), develops no ISR when WCS417r bacteria were applied to the roots, but showed normal levels of ISR when WCS417r bacteria were infiltrated into the leaves. If ET signaling were required only for expression of ISR at the site of challenge inoculation, *eir1-1* plants would develop normal levels of ISR in the leaves after application of WCS417r to the roots. However, this was not the case. Thus, ET signaling is required locally at the site of application of the inducer, and may be involved in the generation or translocation of the systemically transported signal. Here we demonstrated that WCS417r-induced expression of *MYB72* is not regulated by ET, because mutant *ein2-1* plants accumulated normal levels of *MYB72* transcripts in the roots upon treatment with WCS417r (Fig. 2.7B). In addition, the expression of *MYB72* was not activated upon treatment of the roots with ACC (Fig. 2.7A). All together these results demonstrate that MYB72 either acts upstream of ET, or is co-required with components from the ET signaling pathway during the onset of ISR in the roots.

MYB72 is not sufficient for the onset of ISR

Although MYB72 is required for the onset of ISR, overexpression of the *MYB72* gene did not result in enhanced disease resistance (Fig. 2.8B). Hence, another signaling component is likely to be co-required for the expression of ISR. Yeast two-hybrid experiments revealed that MYB72 physically interacts with EIL3, a member of the EIN3 family of transcription factors (Fig. 2.9A). EIN3 and its closest paralogs, the EIN3-like proteins EIL1 and EIL2, are key transcription factors of ET-regulated gene expression and act as positive regulators of ET signaling (Stepanova & Ecker, 2000; Tieman *et al.*, 2001; Guo & Ecker, 2004). They bind to promoters of ET-responsive genes, such as the regulatory gene *ERF1* (*ETHYLENE RESPONSE FACTOR 1*), and initiate a transcriptional cascade leading to the expression of ET-targeted genes (Chao *et al.*, 1997; Solano *et al.*, 1998). If EIL3 would function similarly as EIN3, EIL1 and EIL2 in ET signaling, then physical interaction with MYB72 may facilitate the so far unidentified ET-signaling event that is co-required with MYB72 in the roots for the onset of WCS417r-mediated ISR in the leaves. Besides a potential role in ET signaling, EIL3 (also called SLIM1 for SULFUR LIMITATION 1) was recently shown to function as an important transcriptional regulator in the response of *Arabidopsis* to sulfur deprivation (Maruyama-Nakashita *et al.*, 2006). Collectively, these data highlight that both MYB72 and EIL3 are part of the signaling network involved in the plant's response to biotic and abiotic stresses. Analysis of the putative interaction between MYB72 and EIL3 *in vivo* and its significance for the onset of ISR will shed new light on ISR signaling and is a major challenge for future research.

Model for ISR signal transduction

The identification of MYB72 as an important signaling component in the roots for the systemic onset of ISR adds a new factor to ISR signal transduction. Figure 2.10 summarizes our current understanding of the ISR signaling pathway. The local onset of WCS417r-mediated ISR in the roots requires responsiveness to ET (Knoester *et al.*, 1999), and is associated with an ET-independent activation of the *MYB72* gene. MYB72 is required but not sufficient for the onset of ISR. Hence, MYB72 is assumed to act in concert with another signaling component. MYB72 interacts with EIL3 in vitro, suggesting that both interaction partners are important for the onset of ISR. Systemically in the leaves, expression of ISR requires responsiveness to both JA and ET and is dependent on NPR1 (Pieterse *et al.*, 1998). The induced state of WCS417r-mediated ISR is not associated with major changes in defense-related gene expression (as opposed to SAR) (Verhagen *et al.*, 2004). Instead, ISR-expressing plants are primed to express a specific set of JA/ET-responsive genes faster and to a higher level upon pathogen infection (Van Wees *et al.*, 1999; Hase *et al.*, 2003; Verhagen *et al.*, 2004). This enhanced defensive capacity allows the plants to respond faster and/or more strongly to attackers that trigger JA/ET-dependent defense responses, without major metabolic changes in the absence of an intruder (Conrath *et al.*, 2002; 2006). Therefore, ISR forms

a low-cost defense strategy that is active against a broad spectrum of attackers (Van Hulten *et al.*, 2006).

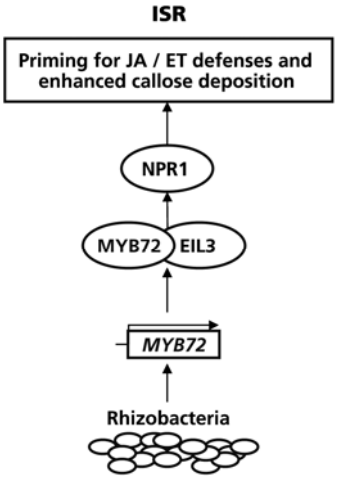

Figure 2.8. Proposed model for the role of MYB72 in the signal transduction pathway controlling rhizobacteria-mediated ISR.

Colonization of the roots by ISR-inducing *P. fluorescens* WCS417r leads to a local, ET-independent activation of the transcription factor gene MYB72. Subsequently, MYB72 interacts physically with the transcription factor EIL3. Downstream of, or in parallel with MYB72/EIL3, a so far unidentified ET signaling component is required in the roots for the onset of broad-spectrum ISR in the leaves. Systemically, the ISR signal transduction cascade requires responsiveness to both JA and ET, and is dependent on NPR1. Finally, induction of ISR is associated with priming for enhanced expression of a large set of JA- and ET-responsive genes that becomes apparent only after pathogen attack. This allows the plant to react more effectively to an invading pathogen, which may explain the broad-spectrum characteristic of rhizobacteria-mediated ISR.

EXPERIMENTAL PROCEDURES

Cultivation of rhizobacteria and pathogens

Non-pathogenic, rifampicin-resistant *Pseudomonas fluorescens* WCS417r and *Pseudomonas putida* WCS358r bacteria were used for induction of ISR (Van Wees *et al.*, 1997). Both strains were grown for 24 h at 28°C on King's medium B (KB) agar plates (King *et al.*, 1954), as described previously (Pieterse *et al.*, 1996). An avirulent strain of *Pseudomonas syringae* pv. *tomato* DC3000, carrying the avirulence gene *avrRpt2* (*Pst* DC3000 (*avrRpt2*) (Kunkel *et al.*, 1993), was used for SAR induction. *Pst* DC3000 (*avrRpt2*) bacteria were grown overnight at 28°C in liquid KB medium supplemented with 25 mg.ml^{-1} kanamycin to select for the plasmid. Virulent *Pst* DC3000 (Whalen *et al.*, 1991) was used for challenge inoculations and cultivated in a similar manner in liquid KB medium without kanamycin. After centrifugation for 10 min at 5,000 x g, the bacterial cells were resuspended in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands) to a final density of $2.5x10^7$ cfu.mL^{-1}.

Hyaloperonospora parasitica strain WACO9 was maintained on susceptible Col-0 plants as described by Koch & Slusarenko (1990). Sporangia were obtained by washing leaves that were densely covered by sporangiophores in distilled water, collected by centrifugation, and resuspended in water to a final density of $5x10^4$ cfu.mL^{-1}.

Alternaria brassicicola strain MUCL20297 was grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) plates containing penicillin (100 ppm) and streptomycin (200 ppm) for 2 weeks at 22°C. Conidia were harvested as described by Broekaert *et al.* (1990) and resuspended in water to a final density of $1x10^6$ spores.mL^{-1}.

Botrytis cinerea strain B0510 was grown on half-strenght PDA plates containing penicillin (100 ppm) and streptomycin (200 ppm) for 2 weeks at 22°C. Spores were collected and resuspended in half-strenght potato dextrose broth (Difco Laboratories, Detroit, USA) to a final density of $5.5x10^5$ spores.ml^{-1}. After a 3-h incubation period, the spores were used for inoculation of plants as described (Thomma *et al.*, 1998).

Preparation of crude cell wall material of WCS417r

To check the effect of killed rhizobacterial cells on the induction of ISR, bacterial cells were collected as described above and resuspended in 50 mM Tris-HCl, 2 mM EDTA (pH 8.5). Subsequently, the cells were sonicated eight times for 15 s on ice at resonance amplitude. Still intact cells were removed by centrifugation at 600 x g for 20 min. After centrifugation of the supernatant at 8,000 x g for 60 min, the pellet of crude cell wall material was resuspended in 10 mM phosphate-buffered saline (pH7.2) containing 0.01% sodium azide, and stored at -80°C until further use.

Plant growth conditions

Seeds of wild-type *Arabidopsis thaliana* Col-0, mutants *ein2-1* (Guzmán & Ecker, 1990), *npr1-1* (Cao *et al.*, 1994), *pad3-1* (Zhou *et al.*, 1999), *myb72-1* (SAIL_713G10), *myb72-2* (SALK_052993), the double mutant *myb72-1/pad3-1*, the transgenic lines 35S::MYB72-OX1 to OX7, and the corresponding empty vector control line EV, were sown in quartz sand. Two-week-old seedlings were transferred to 60-ml pots containing a sand/potting soil mixture that had been autoclaved twice for 20 min with a 24-h interval. For ISR bioassays, a suspension of ISR-inducing WCS417r or WCS358r bacteria (10^9 cfu.ml^{-1}) had been mixed thoroughly through the soil to a final density of $5x10^7$ cfu.g^{-1}, as described previously (Pieterse *et al.*, 1996). Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. Plants were cultivated in a growth chamber with a 9-h day (200 µE m^{-2} s^{-1} at 24ºC) and a 15-h night (20ºC) cycle at 70% relative humidity. Plants were supplied with modified half-strength Hoagland nutrient solution (Hoagland & Arnon, 1938) once a week, as described (Pieterse *et al.*, 1996).

Knockout mutants

Homozygous knockout mutants SAIL_713G10 (Sessions *et al.*, 2002), designated *myb72-1*, and SALK_052993 (Alonso *et al.*, 2003), designated *myb72-2*, containing a T-DNA insertion in the *MYB72* gene (At1g56160), were grown as described above. Confirmation

of the T-DNA insert in SAIL_713G10 was obtained by PCR on genomic DNA using T-DNA left border primer T_1 and *MYB72*-specific primers $MYB72_{F1}$ and $MYB72_{R1}$ following a procedure that was described previously (Sessions *et al.*, 2002). Mutant SALK_052993 was checked for the presence of T-DNA using primer T_2, and *MYB72*-specific primers $MYB72_{F2}$ and $MYB72_{R2}$. The primers used are listed in *Supplementary Table 2.1*. The exact insertion sites of the T-DNAs in *myb72-1* and *myb72-2* were determined by DNA sequencing of the PCR products.

Construction of transgenic plants

A fusion product of the *Cauliflower mosaic virus 35S* promoter and the coding region of *MYB72* was created by double-joint PCR as described by Yu *et al.* (2004). In brief, the *35S* promoter and the coding region of *MYB72* were amplified by PCR from a plasmid containing the *35S* promoter, and a plasmid containing a full-length cDNA of *MYB72*, using the primer pairs $35S_F$ and $35S_R$, and $MYB72_{F3}$ and $MYB72_{R3}$, respectively (Supplementary Table 2.1). The resulting PCR fragments were denatured at 95°C for 5 min, annealed at 59°C for 45 sec, and elongated for 3 min at 68°C using *Pfu* polymerase (Promega Benelux BV, Leiden, the Netherlands). Then, primers $35S_F$ and $MYB72_{R3}$ were added, after which 35 cycles of PCR were performed (1 min at 95°C, 45 s at 59°C, 3 min at 72°C). The resulting double-joint *35S::MYB72* fusion product was cloned into the pCR®-Blunt II-TOPO® vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Breda, the Netherlands). From the resulting plasmid, a 1443-bp *Kpn*I-*Eco*RI fragment containing the *35S::MYB72* fusion was cloned into the binary vector pGreenII229 (Hellens *et al.*, 2000). Finally, the *35S::MYB72* fusion was transferred into pGreenII229-G (Hellens *et al.*, 2000) using the *Kpn*I and *Sac*I restriction sites, thereby replacing the *ß-GLUCURONIDASE* (*GUS*) reporter gene and placing the *35S::MYB72* fusion in front of the *35S* terminator sequence. A derivative of the pGreenII229 vector was used as the empty vector (EV) control. Correct construction of the plasmids was verified by DNA sequencing. Subsequently, the binary vectors were transferred to *Agrobacterium tumefaciens* strain C58(pMP90) (Konzc & Schell, 1986) containing helper vector pSoup (Hellens *et al.*, 2000), after which *Arabidopsis* Col-0 plants were transformed according to the floral dip method (Clough & Bent, 1998). Transformants were selected by spraying T1 progeny with BASTA Finale SL14 (Bayer CropScience BV, Mijdrecht, the Netherlands) according to the manufacturer's instructions. The resulting *MYB72* overexpressing lines 35S::MYB72-OX1 to OX7 were selfed and homozygous T3 lines were selected for use in disease resistance assays.

Induction treatments

Induction of ISR with living rhizobacteria was performed by mixing ISR-inducing rhizobacteria through the soil as described above. For tests with killed cells, a crude cell wall preparation of WCS417r bacteria (in 10 mM $MgSO_4$) was mixed through the soil in

a similar manner, using the equivalent of the number of live bacteria introduced to the soil ($5x10^7$ cfu.g^{-1}). Seven days before challenge inoculation, a similar amount of the crude cell wall material was applied to each plant as a soil drench as described previously (Van Wees *et al.*, 1997).

Biological induction of SAR was performed three days before challenge inoculation by pressure infiltrating three lower leaves with a suspension of *Pst* DC3000(*avrRpt2*) bacteria at 10^7 cfu.ml^{-1}, as described (Pieterse *et al.*, 1996).

For chemical induction of resistance, treatments were performed seven and four days prior to challenge inoculation with *Pst* DC3000, or three days before challenge with *H. parasitica* WACO9. For the *Pst* DC3000 bioassays, the soil was drenched with 10 ml of either 100 µM MeJA, 1 mM ACC, or 1 mM SA. For the *H. parasitica* bioassays, the soil was drenched with 6 ml of 300 µM BTH. Control plants were treated with an equal volume of water. MeJA was purchased from Serva Brunschwig (Amsterdam, the Netherlands), ACC from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands), SA from Malinckrodt Baker BV (Deventer, the Netherlands), and BTH (BION) from CIBA-GEIGY GmbH (Frankfurt, Germany).

*P. syringa*e pv. *tomato* DC3000 bioassays

Plants were challenged when five weeks old by dipping the leaves for 2 s in a solution of 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77 containing $2.5x10^7$ cfu.ml^{-1} *Pst* DC3000 bacteria. One day before challenge inoculation, the plants were placed at 100% relative humidity. Four days after challenge, disease severity was assessed by determining the percentage of diseased leaves per plant. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. Based on the number of diseased and non-diseased leaves, the disease index was calculated for each plant (n=20) as described (Pieterse *et al.*, 1996).

H. parasitica bioassays

Three-week-old *Arabidopsis* Col-0, *npr1-1* and *myb72-1* plants were misted with a *H. parasitica* spore suspension containing $5x10^4$ sporangiospores.mL^{-1}. Inoculated plants were maintained at 17°C and 100% relative humidity for 24 h. Subsequently, humidity was lowered to ambient level to reduce the chance of secondary infections by opportunistic pathogens. Seven days after challenge inoculation humidity was again raised to 100% to induce sporulation. Disease symptoms were scored at nine days after inoculation for about 250 leaves per treatment. Disease ratings were expressed as

severity of disease symptoms and pathogen sporulation on each leaf: I, no sporulation; II, trailing necrosis; III, < 50% of the leaf area covered by sporangia; IV, > 50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse.

Quantification of callose deposition was performed as described by Ton *et al.* (2005). In short, leaves were collected at two days after inoculation and incubated overnight in 96% ethanol. Destained leaves were washed in 0.07 M phosphate buffer, pH 9, incubated for 15 min in 0.07 M phosphate buffer containing 0.005% Calcofluor (fluorescent brightener; Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and 0.01% aniline blue (water blue; Merck, Darmstadt, Germany), and subsequently washed in 0.07 M phosphate buffer containing only 0.01% aniline blue to remove excess Calcofluor. Inspection was performed with an epifluorescence microscope containing an UV filter (bandpass 340 to 380 nm, long-path 425 nm). Callose formation (see inset Fig. 2.4) was quantified as the proportion of attempted penetration sites per leaf containing callose depositions (n = 150).

A. brassicicola bioassays

Because *Arabidopsis* Col-0 is resistant to *A. brassicicola*, whereas camalexin-deficient mutant *pad3-1* (Zhou *et al.*, 1999) is susceptible (Thomma *et al.*, 1999; Ton *et al.*, 2002b), the role of MYB72 was investigated in the double mutant *pad3-1/myb72-1*,created through genetic crossing. When five weeks old, homozygous *pad3-1* and *pad3-1/myb72-1* plants (*n*=20) were challenge inoculated with *A. brassicicola* by applying 3-µL droplets of water containing $1x10^6$ spores.ml^{-1} onto the second, third and fourth true leaf pair of each plant. Inoculated plants were kept at 100% relative humidity. At five days after challenge, disease severity was determined. Disease rating was expressed on the basis of symptom severity: I, no visible disease symptoms; II, non-spreading lesion; III, spreading lesion without tissue maceration; IV, spreading lesion with tissue maceration and sporulation of the pathogen.

B. cinerea bioassays

Five-week-old Col-0 and *myb72-1* plants (*n*=20) were challenge inoculated with *B. cinerea* by applying 5-µL droplets of the spore suspension onto fresh needle-prick wounds on the second, third and fourth true leaf pair of each plant as described (Thomma *et al.*, 1998). Inoculated plants were kept at 100% relative humidity. At five days after challenge, disease severity was determined. Disease ratings were expressed as the percentage of leaves showing spreading lesions.

Root colonization

Colonization of the rhizosphere of wild-type and mutant plants by rifampicin-resistant WCS417r and WCS358r bacteria was examined at the end of each ISR bioassay. In quadruplicate, roots of five plants per treatment were harvested, weighed, and shaken vigorously for 1 min in 5 ml of 10 mM $MgSO_4$ containing 0.5 g of glass beads (0.17

mm). Appropriate dilutions were plated onto KB agar supplemented with cycloheximide (100 mg.l^{-1}), ampicillin (50 mg.l^{-1}), chloramphenicol (13 mg.l^{-1}), and rifampicin (150 mg.l^{-1}), which is selective for rifampicin-resistant, fluorescent *Pseudomonas* spp. (Geels & Schippers, 1983). After overnight incubation at 28ºC, the number of rifampicin-resistant colony-forming units (cfu) per gram of root fresh weight was determined.

RNA extraction and northern blot analysis

Total RNA was extracted by homogenizing frozen tissue in extraction buffer (0.35 M glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 ml per gram plant tissue). The homogenates were extracted with phenol/chloroform/isoamylalcohol (25:24:1) and the RNA was precipitated using LiCl, as described previously (Sambrook *et al.*, 1989). For northern blot analysis, 10 µg RNA was denatured using glyoxal and DMSO (Sambrook *et al.*, 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto a Hybond-N^+ membrane (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. Northern blots were hybridized with gene-specific probes for *MYB72* (At1g56160), similarly as described by Pieterse *et al.* (1998).

Quantitative Real-Time PCR

Total RNA was cleaned using RNeasy Plant Mini Kit columns (Qiagen Benelux BV, Venlo, the Netherlands). Analysis of gene expression in the roots was performed by Q-PCR, basically as described by Czechowski *et al.* (2004). Two µg of RNA was digested with Turbo DNA-free™ (Ambion, Huntingdon, United Kingdom) according to the manufacturer's instructions. To check for genomic DNA contamination, a PCR with primers designed on *EIL2* (At5g21120; $EIL2_F$ and $EIL2_R$) was carried out. DNA-free total RNA was converted to cDNA using oligo-dT20 primers (Invitrogen, Breda, the Netherlands), 10 mM dNTPs, and SuperScript™ III Reverse Transcriptase (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Efficiency of cDNA synthesis was assessed by Q-PCR, using primers of the constitutively expressed gene *UBI10* (At4g05320; $UBI10_F$ and $UBI10_R$). Primers for *MYB72* (At1g56160; $MYB72_{F5}$ and $MYB72_{R5}$) and the ET-responsive gene *EBF2* (At5g25350; EBF2-F and EBF2-R) were designed and checked as described by Czechowski *et al.* (2004). Nucleotide sequences of all primers are given in *Supplementary Table 2.1*.

Q-PCR analysis was performed in optical 96-well plates with a MyIQ™ Single Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands), using SYBR® Green to monitor dsDNA synthesis. Each reaction consisted of 1 µL of cDNA, 0.5 µL of each of the two gene-specific primers (10 pmol.μL^{-1}), and 3.5 µL 2x IQ SYBR® Green Supermix reagent (Bio-Rad, Veenendaal, the Netherlands) in a final volume of 15 µl. The following PCR program was used for all PCR reactions: 95°C for 3 min; 40 cycles of

95°C for 30 sec, 59.5°C for 30 s, and 72°C for 30 s. C_T (threshold cycle) values were calculated using Optical System Software, version 1.0 for MyIQTM (Bio-Rad, Veenendaal, the Netherlands). Subsequently, C_T values were normalized for differences in dsDNA synthesis, using those of the constitutively expressed reference gene At1g13320 as described (Czechowski *et al.*, 2005), after which the fold-differences in transcript levels were calculated.

Yeast two-hybrid assays

Constructs for yeast two-hybrid analyses were generated using vectors pDESTTM32 and pDESTTM22 (Invitrogen, Breda, the Netherlands) for protein fusions to the GAL4 DNA-binding domain (BD) or transcriptional-activation domain (AD), respectively. Full-length coding regions of *MYB72* and *EIL3* cDNA were introduced in both vectors using the GATEWAY™ technology (Invitrogen), following the manufacturer's instructions. Clones containing the BD::MYB72, AD::MYB72, BD::EIL3, and AD::EIL3 fusions were checked by sequence analysis and subsequently used in the yeast two-hybrid assay. AD and BD plasmids were transformed into the **a** and **α** mating types of *Saccharomyces cerevisiae* strain PJ69-4A, using a lithium acetate/polyethylene glycol protocol described by Gietz & Woods (2006). PJ69-4A carries *ADE2*, *HIS3*, *URA* and *LacZ* reporters for reconstituted GAL4 activity (James *et al.*, 1996). Transformants were selected on yeast selective drop-out medium (SD) lacking either leucine (-leu) for selection of the BD vectors, or tryptophan (-trp) for selection of the AD vectors. Opposite mating types were co-cultured overnight on nutrient-rich YAPD medium (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) at 30°C. Diploids harboring both plasmids were selected on SD medium lacking both leucine and trypthophan (-leu, -trp) and used in the yeast two-hybrid assay. Autoactivation levels of yeast transformants harboring either BD::MYB72 or BD::EIL3 were determined using SD medium lacking leucine, adenine, uracil and histidine (-leu, -ade, -ura, -his), to which 0, 10, 25, 50, 75 or 100 mM of the histidine biosynthesis inhibitor 3-amino-1,2,4-triazole (3AT) was added (Durfee *et al.*, 1993). Since autoactivation could not be suppressed fully when using 100 mM 3AT, only the latter concentration was used in the assay. The yeast two-hybrid assay of interactions between MYB72 and EIL3 was performed by growing the yeast strains with the different two-hybrid combinations on selective SD medium (-leu, -trp, -ade, -ura, -his, +3AT) for 3 days at 20°C to reduce background growth levels.

ACC-induced triple response

Seeds of *Arabidopsis* were surface sterilized for five min in 5% (v/v) sodium hypochlorite, washed in 70% (v/v) ethanol, and air-dried. Seeds were subsequently distributed evenly on 1.0% (w/v) agar medium (pH 5.7) containing 0.5% (w/v) Murashige & Skoog (MS) salts (Duchefa bv, Haarlem, The Netherlands), 0.5% (w/v) sucrose, and different concentrations of filter-sterilized ACC, which was added from a 10 mM stock solution. The effect of ACC-derived ET on hypocotyl and primary root length in etiolated seedlings was determined essentially according to Guzmán & Ecker (1990). After pre-germination in the dark for two days at 4°C, seedlings were grown for an additional three to seven days at 20°C in darkness after which the triple response was monitored.

SUPPLEMENTARY MATERIAL

Supplementary Figure 2.1. Morphological phenotype of transgenic 35S::MYB72 lines. Growth of Col-0, empty vector control (EV), knockout mutant *myb72-1*, and transgenic 35S::MYB72 lines OX1 to OX7 was compared by measuring the diameter of the rosettes of plants that were 21, 28, and 35 days old (n=20).

Supplementary Figure 2.2. Alignment of ATMYB72 with other R2R3 MYB transcription factor proteins.

Supplementary Table 2.1. Nucleotide sequences of the primers used in this study.

All supplementary materials can be downloaded from:
http://www.bio.uu.nl/~fytopath/GeneChip_data.htm

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CHAPTER 3

Signaling of *Trichoderma*-induced systemic resistance in *Arabidopsis* is similar to rhizobacteria-mediated ISR

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ABSTRACT

Selected root-colonizing bacteria and fungi trigger broad-range systemic resistance in an array of plant species. *Trichoderma* spp. have also been demonstrated to enhance the defensive capacity of various plant species. The signaling pathway underlying *Trichoderma*-mediated resistance appears to show similarities to both systemic acquired resistance (SAR) and rhizobacteria-mediated induced systemic resistance (ISR), as root colonization by this fungus was demonstrated to be accompanied by changes in SA and JA signaling. To unravel the signal-transduction pathway of *Trichoderma*-induced resistance, we studied the responses of the model plant *Arabidopsis thaliana* to root colonization by *T. asperellum* T34. The fungus remained confined to the roots but rendered the leaves more resistant to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*), the biotrophic oomycete *Hyaloperonospora parasitica*, and the necrotrophic fungus *Plectosphaerella cucumerina*. The induced systemic resistance against *P. syringae* pv. *tomato* was lost in the *myb72* and *npr1* mutants of *Arabidopsis* that are impaired in rhizobacteria-mediated ISR, but fully retained in the *sid2* mutant that is deficient in SAR. As reported for *P. fluorescens* WCS417r-induced ISR, treatment with T34 primed the expression of defense genes responsive to jasmonic acid, as well as the formation of callose-containing papillae at sites of pathogen entry. Thus, the systemic resistance induced by *Trichoderma asperellum* T34 in *Arabidopsis* is similar to rhizobacteria-mediated ISR.

INTRODUCTION

For health and environmental reasons, there is an increasing legislative pressure to reduce reliance on pesticides for disease control in agriculture. This has raised the need to study alternatives, such as biological control agents. *Trichoderma* spp. are cosmopolitan soil fungi, widely used to interfere with plant pathogens and pests. It is well established that their effectiveness results from different modes of action. Firstly, *Trichoderma* spp. compete with other soil micro-organisms for nutrients in the rhizosphere (Chet, 1987). Secondly, they can act directly on other soil inhabitants by producing a wide range of antibiotic substances (Schirmböck *et al.*, 1994) and lytic enzymes to parasitize other fungi (Harman *et al.*, 1981; Lorito *et al.*, 1996; Woo *et al.*, 1999). Thirdly, *Trichoderma* spp. are able to inhibit or degrade pectinases and other enzymes that are essential for the invasive activity of plant-pathogenic fungi (Zimand *et al.*, 1996). Finally, apart from these direct effects on plant pathogens, recent findings suggest that *Trichoderma* spp. can systemically elevate the plant's resistance against various pathogens, including fungi and bacteria (De Meyer *et al.*, 1998; Shoresh *et al.*, 2005). Root colonization by some strains of *Trichoderma* spp. results in the induction of systemic resistance against different attackers in various plant species, as shown by spatial separation between biocontrol agent and plant attacker (Harman *et al.*, 2004a).

Enhancement of basal resistance levels is a common reaction of plants to biotic and abiotic stresses (Van Loon, 2000), and is commonly referred to as induced resistance. The classic example is that of systemic acquired resistance (SAR) (reviewed in Durrant & Dong, 2004). Initial attack by a pathogen not only triggers local defense responses, but can also lead to the generation of a signal that is spread throughout the plant. Upon perception of this signal, the distal plant parts become more resistant against subsequent attack by a broad range of pathogens. SAR depends on the production of, and responsiveness to, salicylic acid (SA) (Delaney *et al.*, 1994; Mauch-Mani & Métraux, 1998; Nawrath & Métraux, 1999) and is associated with the induction of novel PATHOGENESIS-RELATED (PR) proteins (Van Loon & Van Strien, 1999). In *Arabidopsis*, pathogen-induced SA is synthesized from isochorismate by the enzyme isochorismate synthase, which is encoded by the *SID2* (*SALICYLIC ACID DEFICIENT* 2) gene (Wildermuth *et al.*, 2001). Although SA is necessary, it is not the transported signal (Vernooij *et al.*, 1994) , the nature of which is still unknown. Other crucial nodes in SAR signaling are the methyl esterase SABP2 (SALICYLIC ACID BINDING PROTEIN 2) and the transcriptional co-activator NPR1 (NON-EXPRESSOR OF PR-GENES 1) (Kumar & Klessig, 2003; Dong, 2004; Forouhar *et al.*, 2005).

The phenotypically similar induced systemic resistance (ISR) that is triggered upon root colonization by specific non-pathogenic, root-colonizing bacteria depends on a different signal transduction cascade (Pieterse *et al.*, 1996). Although functional NPR1 is also necessary, responsiveness to jasmonic acid (JA) and ethylene (ET), rather than SA signaling, is required for ISR (Pieterse *et al.*, 1998). Even though colonization of the roots by ISR-inducing bacteria enhances the plant's resistance level, no defense mechanisms

become activated in the above-ground tissue (Van Wees *et al.*, 1999; Verhagen *et al.*, 2004). Yet, these tissues respond faster and more strongly to pathogen attack (Van Wees *et al.*, 1999; Hase *et al.*, 2003; Verhagen *et al.*, 2004), a phenomenon known as priming (Conrath *et al.*, 2006). Locally in the roots, bacterization does alter the expression of over 90 genes (Verhagen *et al.*, 2004). Recently, transcriptional activation of one of these, the transcription factor gene *MYB72*, has been demonstrated to be crucial for ISR in *Arabidopsis* (Chapter 2).

T. asperellum isolate T203 has been shown to induce resistance in cucumber against the bacterial leaf pathogen *Pseudomonas syringae* pv. *lachrymans* (Shoresh *et al.*, 2005). Upon colonization of cucumber roots by T203, no differences in host SA or ET production could be observed (Shoresh *et al.*, 2005). However, blocking the action of ET or the synthesis of JA with the chemical inhibitors silver thiosulfate and diethyldithiocarbamate, respectively, diminished the enhanced protective effects. This suggests a similar signaling role for these hormones in *Trichoderma*-mediated systemic resistance in cucumber (Shoresh *et al.*, 2005), as in rhizobacteria-mediated resistance in *Arabidopsis*.

T. asperellum isolate T34 is a biocontrol agent useful to control diseases produced by soilborne pathogens as *Fusarium oxysporum* (Cotxarrera *et al.*, 2002) and *Rhizoctonia solani* (Trillas *et al.*, 2006). Furthermore, Segarra *et al.* (2007) demonstrated that T34 is able to reduce *P. syringae* pv. *lachrymans* growth on cucumber leaves. Contrary to the effect of biocontrol bacteria, root colonization by *Trichoderma* transiently increased peroxidase (Yedidia *et al.*, 2003; Segarra *et al.*, 2007) and chitinase (Yedidia *et al.*, 2003) activity in local, as well as systemic tissues. There is evidence that high doses of *Trichoderma* may additionally activate the SAR pathway: unlike treatment with the ISR-inducing rhizobacterial strain *Pseudomonas fluorescens* WCS417r, application of high densities of T34 spores resulted in direct increases of JA and SA levels (Segarra *et al.*, 2007). Thus, both similarities and differences seem to exist between the signaling pathways underlying rhizobacteria- and *Trichoderma*-induced resistance. However, the results are difficult to compare, because they were obtained using different plant species.

In order to compare the systemic resistances mediated by *Trichoderma* spp. with rhizobacteria, we tested the ability of *T. asperellum* T34 to induce systemic resistance against various foliar diseases in the model plant *Arabidopsis*. Furthermore, the signaling pathway involved was analyzed by using *Arabidopsis* mutants impaired in SAR, ISR or both.

RESULTS

Trichoderma triggers systemically induced resistance against *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis*

Trichoderma spp. have been demonstrated to systemically induce resistance in different di- and monocotyledonous plants against diverse attackers (Harman *et al.*, 2004a). To

determine whether root colonization by *T. asperellum* T34 (T34) can enhance resistance in *Arabidopsis* against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), the severity of bacterial speck disease inflicted by this pathogen were quantified for control (Ctrl) and T34-treated plants. As shown in Figure 3.1, plants treated with T34 showed significantly fewer disease symptoms than those pre-treated with a control solution. The extent of the T34-mediated reduction in disease severity was larger than that conferred by the ISR-mediating rhizobacterial strain *P. fluorescens* WCS417r. This difference in effectiveness could be caused by local effects if T34 would colonize *Arabidopsis* shoots endophytically, or be truly systemic if T34 remains confined to the roots. These two possibilities were further investigated.

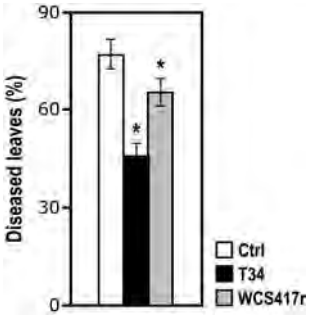

Figure 3.1. *Trichoderma* triggers systemic protection against *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis*.

Levels of T34- and WCS417r-induced protection against *Pst* DC3000 in *Arabidopsis* Col-0. Resistance was induced by growing the plants for three weeks in soil containing either T34 conidia or ISR-inducing WCS417r bacteria. Five-weeks-old plants were challenge inoculated with a bacterial suspension of virulent *Pst* DC3000. Four days after challenge inoculation, the percentage of diseased leaves was assessed. Asterisks indicate statistically significant differences compared to non-induced control plants (Student's *t*-test, $\alpha<0.05$, *n*=20).

Trichoderma-induced resistance is systemic

Rhizosphere-competent *Trichoderma* spp. are able to penetrate and colonize the epidermis and outer root cortex of cucumber seedlings (Yedidia *et al.*, 1999; Harman, 2000). However, an endophytic *Trichoderma* species (*T. stromaticum*) (Evans *et al.*, 2003) has also been described. If T34 could colonize *Arabidopsis* in a similar manner, the observed partial prevention of disease development (Fig. 3.1) could also be a result of locally induced defense mechanisms. To examine this possibility, externally sterilized sections of *Arabidopsis* stems were harvested at intervals after T34 root colonization, and placed on *Trichoderma*-specific agar medium. None of the stem sections gave rise to outgrowth of *Trichoderma* mycelium (data not shown). In contrast, T34 spores were perfectly able to germinate and grow when placed on the same medium. These observations demonstrate that T34 treatment of *Arabidopsis* roots does not lead to spread of the fungus into the above-ground parts. Hence, the observed increase in resistance of T34-treated plants does not arise from locally, but rather from systemically induced resistance.

Trichoderma-induced resistance shares pathway signaling with rhizobacteria-mediated induced systemic resistance

To elucidate whether the systemic resistance induced by T34 is similar to SAR, to ISR, or to both, the *sid2-2* and the *myb72-1* mutants of *Arabidopsis* were tested, as well as *npr1-1*. Colonization of wild-type *Arabidopsis* roots by T34 resulted in a 26% reduction in disease symptoms compared to control plants. Under the same conditions, a 24% was observed in the *sid2-2* mutant, which is unable to produce SA, whereas no significant reduction was evident in the *myb72-1* or *npr1-1* mutants that are impaired in ISR, and both ISR and SAR, respectively. Thus, the *Trichoderma*-induced systemic resistance was fully retained in the SAR mutant *sid2-2*, but lost in the ISR mutant *myb72-1*. Furthermore, treatment with T34 did not lead to induction or priming of the SAR marker gene *PR-1* (data not shown). These results demonstrate that the systemic resistance induced by *Trichoderma* is similar to rhizobacteria-mediated ISR.

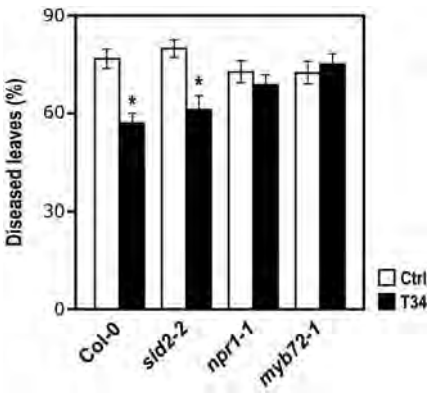

Figure 3.2. *Trichoderma*-induced systemic resistance depends on NPR1 and MYB72.

Levels of T34-induced protection against *Pst* DC3000 in wild-type Col-0 and *sid2-2*, *npr1-1*, and *myb72-1* mutant *Arabidopsis* plants. Induction of resistance, pathogen challenge and disease assessment were performed as described in the legend to Figure 1. Asterisks indicate statistically significant differences compared to non-induced control plants (Students *t*-test, α<0.05, *n*=20).

Trichoderma-mediated ISR is effective against different types of pathogens

To investigate whether T34-mediated ISR, like WCS417r-elicited ISR, is effective against a broad range of pathogens, disease severity of T34- or Ctrl-treated wild-type plants was determined upon challenge inoculation with the biotrophic oomycete *Hyaloperonospora parasitica* and the necrotrophic fungus *Plectosphaerella cucumerina*. Figure 3.3A shows that T34-treated plants developed less *H. parasitica*-inflicted mildew symptoms compared to the ones treated with control solution. Similarly, root colonization by T34 resulted in less necrosis when *P. cucumerina* was used as the challenging pathogen (Fig. 3.3B). Together with the data on the enhanced protection against *Pst* DC3000 (Fig. 3.1), these results demonstrate that root colonization by T34 triggers resistance against diverse attackers of *Arabidopsis* leaves.

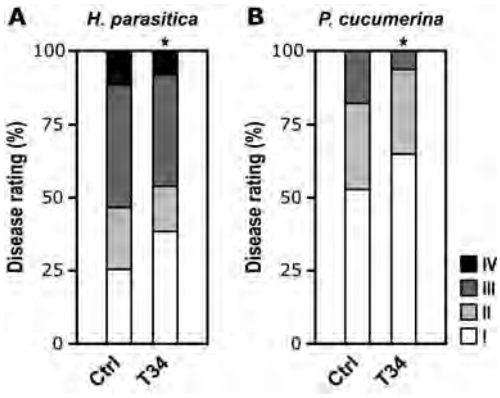

Figure 3.3. *Trichoderma*-mediated systemic resistance has a broad range of effectiveness.

(A) **Quantification of ISR in *Arabidopsis* Col-0 against *H. parasitica*. Resistance was induced by growing the plants in soil containing T34 conidia. Plants were challenge inoculated with *H. parasitica* when three weeks old. Disease severity was determined nine days after challenge. Disease ratings are expressed as the percentage of leaves (n=~250) in disease-severity classes: I, no sporulation; II, trailing necrosis; III, < 50% of the leaf area covered with sporangia; IV, > 50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse.** (B) **Quantification of T34-mediated resistance against *P. cucumerina*. ISR was induced as described above. Plants were inoculated with *P. cucumerina* when five weeks old. At seven days after challenge, disease severity was determined. Disease ratings were expressed as severity of disease symptoms on each leaf: I, no symptom; II, lesion diameter ≤ 2 mm; III, lesion diameter ≥ 2 mm. Asterisks indicate statistically significantly different distributions of the disease severity classes compared with the non-induced control treatments (Chi-square, α<0.05, *n*=120).**

Trichoderma primes for defense responses upon challenge inoculation

An early defense response of *Arabidopsis* to attack by *H. parasitica* is the formation of callose-containing cell-wall appositions at sites of attempted penetration. Several resistance-inducing treatments have been described to boost this papillae formation, amongst which root colonization by WCS417r bacteria (Chapter 2; Kohler *et al.*, 2002; Ton *et al.*, 2005). Figure 3.4A shows that pre-treatment with T34 also leads to a higher percentage of germinating *H. parasitica* spores being blocked by the formation of callose-containing papillae.

Treatment with WCS417r also primes for enhanced transcriptional activity of JA- and ET-responsive genes (Van Wees *et al.*, 1999; Verhagen *et al.*, 2004). Notably, expression of the JA-responsive gene *LOX2* (*LIPOXYGENASE 2*) serves as a molecular marker for WCS417r-induced priming for defense in ISR (Chapter 5; Conrath *et al.*, 2006). As shown in Figure 3.4B, treatment with T34 likewise resulted in an augmented *LOX2* gene expression. These results confirm that the systemic resistance induced as a result of root colonization by T34 is similar to ISR. Analogous to *P. fluorescens* WCS417r (Chapter 2; Van Wees *et al.*, 1999; Verhagen *et al.*, 2004), T34 primes defense responses in the systemic plant parts to be expressed faster and more strongly upon pathogen attack.

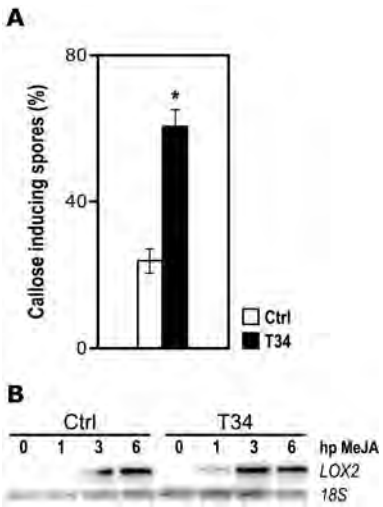

Figure 3.4. *Trichoderma* primes for enhanced defense responses upon challenge inoculation.

(A) **Induced resistance against *H. parasitica* is associated with enhanced deposition of callose-containing papillae at sites of attempted penetration, resulting in a reduction of the number of spores that lead to successful penetration into *Arabidopsis* leaves. Two days after challenge with *H. parasitica*, leaves of plants of which the roots were pre-treated with water (Ctrl) or T34 were stained with Calcofluor/aniline blue and analyzed by epifluorescence microscopy (UV), and the percentage of germinating spores that led to callose deposition in the epidermal cell layer was determined.**

(B) ***LOX2* expression in five-weeks-old Ctrl- and T34-treated plants at different times after treatment (hp) with 100 µM MeJA. Equal loading of RNA samples was checked by using a probe for the constitutively expressed 18S rRNA (*18S*).**

DISCUSSION

Birgirimana *et al.* (1997) showed that treating soil with *Trichoderma harzianum* isolate T39 made leaves of bean plants resistant to the rot diseases caused by the fungal pathogens *Botrytis cinerea* and *Colletotrichum lindemuthianum*, even though T39 was present only on the roots and not on the foliage. Since then, several *Trichoderma* spp. have been claimed to induce local and/or systemic resistance to plant pathogens in diverse plant species, such as bean, cotton, cucumber, and maize (Bigirimana *et al.*, 1997; Howell *et al.*, 2000; Yedidia *et al.*, 2003; Harman *et al.*, 2004b; Shoresh *et al.*, 2005). Here we described that application of *T. asperellum* isolate T34 to the roots of *Arabidopsis* plants makes leaves of the plant more resistant to the bacterial pathogen *Pst* DC3000 (Fig. 3.1), the biotrophic oomycete *H. parasitica* (Fig 3.3A), and the necrotrophic fungus *P. cucumerina* (Fig. 3.3B).

The pathway by which *Trichoderma*-induced resistance is established, has not been thoroughly investigated so far. Next to a requirement of a specific *Trichoderma*-induced MAPK protein (Shoresh *et al.*, 2006), the phytohormones JA and ET have been suggested to be involved in the induction of systemic defense by *T. asperellum* T203 against *P. syringae* pv. *lachrymans* in cucumber (Shoresh *et al.*, 2005). When the synthesis of JA or ET by the plant was inhibited, the induction of resistance by T203 was reduced or lost. Responsiveness to these hormones is also required for rhizobacteria-mediated ISR (Pieterse *et al.*, 2000). Pre-treatment of *Arabidopsis* with ISR-inducing *P. fluorescens* WCS417r primes the plants for enhanced expression of JA- and ET-regulated genes, although the levels of these hormones are not altered (Pieterse *et al.*, 2000; Verhagen *et al.*, 2004). Similarly, treatment of cucumber plants with T203 leads to an enhanced

expression of specific defense-related genes, without observable changes in ET or SA production.

Mutant *sid2-2* plants are disrupted in their ability to develop SAR (Nawrath & Métraux, 1999), while rhizobacteria-mediated ISR is still operative (Ton *et al.*, 2002a). Pre-treatment with T34 increased resistance in *sid2-2* plants to the same level as in wild-type Col-0 (Fig. 3.2), demonstrating that SA production does not play a role in *Trichoderma*-induced resistance. On the contrary, we demonstrated that the MYB72 protein is required for eliciting *Trichoderma*-induced resistance against *Pst* DC3000. The transcription factor gene *MYB72* is one of over 90 genes of which the expression is altered locally upon colonization by ISR-inducing *P. fluorescens* WCS417r (Verhagen *et al.*, 2004). Transcriptional activation of this gene is required for the generation of ISR in *Arabidopsis*, as evident from the use of the *myb72-1* and *myb72-2* mutants (Chapter 2). These mutants are not impaired in biologically or chemically induced SAR (Chapter 2). Thus, the signaling pathway underlying *Trichoderma*-induced resistance resembles that of rhizobacteria-mediated ISR.

Generation of systemic resistance does not necessarily require a direct induction of defense responses upon perception of the resistance-inducing signal. Many examples indicate that defense responses are primed for a faster and stronger activation upon challenge (Conrath *et al.*, 2006). This phenomenon of priming has been demonstrated to underlie rhizobacteria-mediated ISR. Colonization of *Arabidopsis* roots by *P. fluorescens* WCS417r did not result in a transcriptional reprogramming in the distal plant parts, but rather primed gene expression for enhanced responsiveness to subsequent pathogen attack (Van Wees *et al.*, 1999; Verhagen *et al.*, 2004). Likewise, the WCS417r-enhanced formation of callose-containing papillae becomes apparent only after stress exposure (Chapter 2).

Priming of defense responses has also been demonstrated for resistance induced by rhizobacteria other than WCS417r. Pre-treatment of *Arabidopsis* plants with the biocontrol agent *Pseudomonas putida* LSW17S, a strain that enhances resistance in this species (Ahn *et al.*, 2007) and in solanaceous crops (Lee *et al.*, 2005) against various pathogens, primed the formation of callose-containing papillae, generation of active oxygen species, and the expression of various defense-related genes for an augmented response after pathogen attack (Ahn *et al.*, 2007). Furthermore, *Serratia marcescens* strain 90-166- or *P. fluorescens* 89B61-induced resistance of cucumber plants against the anthracnose fungus *Colletotrichum orbiculare* was associated with elevated levels of phenolic compounds and callose at sites of fungal penetration (Jeun *et al.*, 2004).

Trichoderma-mediated ISR has been suggested to act through priming as well. Cucumber leaves of plants grown in a potting mix containing *Trichoderma hamatum* T382 did not show any difference in ß-1,3-glucanase activity compared to controls. However, upon attack by *C. orbiculare*, activity was higher when these plants were grown in the potting mix containing T382 (Zhang *et al.*, 1998). Both direct activation and potentiation of defenses were observed upon treatment of cucumber roots with *Trichoderma asperellum* T203 (Shoresh *et al.*, 2005). In contrast to typical ISR, chitinase

and peroxidase activities and mRNAs were increased transiently both locally and systemically (and ß-1,3-glucanase locally) after inoculating the plant with this strain in the absence of pathogens (Yedidia *et al.*, 1999; 2000; Shoresh *et al.*, 2005). Such a direct effect on peroxidase was not observed when cucumber plants were pre-treated with T34 at the standard concentration (10^5 cfu.mL^{-1}), although increases of peroxidase activity were observed when 10^6 or 10^7 cfu.mL^{-1} were applied (Segarra *et al.*, 2007). Besides a transient direct induction of peroxidase activity, T203 treatment resulted in a primed defense reaction to pathogen attack (Shoresh *et al.*, 2005).

In our study we also observed a priming effect as a result of root colonization by T34. Like WCS417r, T34 treatment resulted in a higher percentage of germinating *H. parasitica* spores being blocked by the formation of callose-containing papillae (Fig. 3.4A). Furthermore, MeJA-triggered *LOX2* gene expression was augmented in T34- as compared to control-treated plants (Fig. 3.4B). Priming of JA-responsive genes, such as *LOX2*, is typical for rhizobacteria-mediated ISR (Verhagen *et al.*, 2004; Conrath *et al.*, 2006). Collectively, our results indicate that the signaling pathway by which *Trichoderma*-induced resistance is established is similar to the one that is active in *P. fluorescens* WCS417r-induced ISR and that it leads to priming of similar defense responses.

EXPERIMENTAL PROCEDURES

Cultivation of biocontrol agents and pathogens

Trichoderma asperellum strain T34 (Trillas & Cotxarrera, 2002) was grown on 10 g.l^{-1} malt agar plates for five days at 22°C. Conidia were collected in distilled water and resuspended to a final density of $5x10^6$ colony-forming units (cfu).mL^{-1}.

Non-pathogenic, rifampicin-resistant *Pseudomonas fluorescens* WCS417r bacteria were used for induction of ISR (Van Wees *et al.*, 1997). WCS417r was grown for 24 h at 28°C on King's medium B (KB) agar plates (King *et al.*, 1954), collected and washed by centrifugation as described previously (Pieterse *et al.*, 1996), and resuspended to a final density of 10^9 cfu.mL^{-1}.

Virulent *Pst* DC3000 (Whalen *et al.*, 1991) was grown overnight at 28°C in liquid KB medium. After centrifugation for 10 min at 5,000 x *g*, the bacterial cells were resuspended in 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands) to a final density of $2.5x10^7$ cfu.mL^{-1}.

Hyaloperonospora parasitica strain WACO9 was maintained on susceptible Col-0 plants as described by Koch & Slusarenko (1990). Sporangia were obtained by washing leaves that were densely covered by sporangiophores in distilled water, collected by centrifugation, and resuspended in water to a final density of $5x10^4$ spores.mL^{-1}.

Plectosphaerella cucumerina (Palm *et al.*, 1995), isolated from naturally infected *Arabidopsis*, was grown on 19.5 $g.l^{-1}$ potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) plates for two weeks at 22°C. Conidia were harvested as described by Broekaert *et al*. (1990), and resuspended in 10 mM $MgSO_4$ to a final density of 5×10^6 $spores.mL^{-1}$.

Plant growth conditions

Seeds of wild-type *Arabidopsis thaliana* accession Col-0 and mutants *myb72-1* (Chapter 2), *npr1-1* (Cao *et al.*, 1994), and *sid2-2* (Nawrath & Métraux, 1999) were sown in quartz sand. Two-week-old seedlings were transferred to 60-mL pots containing a sand-potting soil mixture that had been autoclaved for 20 min at 120°C twice on consecutive days. Plants were cultivated in a growth chamber with a 9-h day (200 $\mu E\ m^{-2}\ s^{-1}$ at 24°C) and a 15-h night (20°C) cycle at 70% relative humidity. Plants were watered on alternate days and once a week supplied with modified half-strength Hoagland's nutrient solution as described previously (Pieterse *et al.*, 1996).

Induction treatments

For induced resistance bioassays, the soil was mixed with T34 conidia to a final density of 10^5 $cfu.g^{-1}$, before transplanting the *Arabidopsis* seedlings. Similarly, a suspension of ISR-inducing WCS417r was mixed thoroughly through the soil to a final density of $5x10^7$ $cfu.g^{-1}$, as described previously (Pieterse *et al.*, 1996). Control soil was supplemented with an equal volume of 10 mM $MgSO_4$.

***Pseudomonas syringae* pv. *tomato* DC3000 bioassays**

Plants were challenged when five weeks old by dipping the leaves for 2 s in a solution of 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77, containing $2.5x10^7$ $cfu.mL^{-1}$ *Pst* DC3000 bacteria. One day before challenge inoculation, the plants were placed at 100% relative humidity. Four days after challenge, disease severity was assessed by determining the percentage of diseased leaves per plant. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. Based on the number of diseased and non-diseased leaves, the disease index was calculated for each plant (n=20) as described (Pieterse *et al.*, 1996).

***Hyaloperonospora parasitica* bioassays**

Three-week-old plants were misted with a *H. parasitica* spore suspension containing $5x10^4$ $spores.mL^{-1}$. Inoculated plants were maintained at 17°C and 100% relative humidity for 24 h. Subsequently, humidity was lowered to ambient level to reduce the

chance of secondary infections by opportunistic pathogens. Seven days after challenge inoculation humidity was again raised to 100% to induce sporulation. Disease symptoms were scored at nine days after inoculation on about 250 leaves per treatment. Disease ratings were expressed as severity of disease symptoms and pathogen sporulation on each leaf: I, no sporulation; II, trailing necrosis; III, < 50% of the leaf area covered by sporangia; IV, > 50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse.

Quantification of callose deposition was performed as described by Ton *et al.* (2005). In short, leaves were collected two days after inoculation and incubated overnight in 96% ethanol. Destained leaves were washed in 0.07 M phosphate buffer, pH 9, incubated for 15 min in 0.07 M phosphate buffer containing 0.005% Calcofluor (fluorescent brightener; Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and 0.01% aniline-blue (water blue; Merck, Darmstadt, Germany), and subsequently washed in 0.07 M phosphate buffer containing only 0.01% aniline blue to remove excess Calcofluor. Inspection was performed with an epifluorescence microscope containing a UV filter (bandpass 340 to 380 nm, long-path 425 nm). Callose depositions in response to *H. parasitica* infection were quantified by determining the average percentage of callose-inducing spores per infected leaf (n=15).

Plectosphaerella cucumerina bioassays

Five-week-old plants (n=20) were challenge inoculated with *P. cucumerina* (Palm *et al.*, 1995) by applying 6-µL droplets containing $5x10^6$ spores.mL^{-1} to five fully expanded leaves, as described previously (Ton & Mauch-Mani, 2004). Inoculated plants were kept at 100% relative humidity. At seven days after challenge, disease severity was determined. Disease ratings were expressed as severity of disease symptoms on each leaf: I, no symptoms; II, lesion diameter ≤ 2 mm; III, lesion diameter ≥ 2 mm.

Methyl jasmonate treatment

Treatment with methyl jasmonate (MeJA) was performed by dipping five-week-old plants in an aqueous solution containing 100 µM MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands) and 0.015% Silwet L-77 (Van Meeuwen Chemicals B.V., Weesp, the Netherlands), as described previously (Pieterse *et al.*, 1998). Leaf rosettes were harvested at 0, 1, 3 and 6 h after induction treatment and immediately frozen in liquid nitrogen.

Detection of *Trichoderma* in plant tissue

Sections of *Arabidopsis* stems were taken two and four weeks after transplanting seedlings in soil containing T34. The pieces were surface-sterilized by soaking them in

96% ethanol for 1 min, then in 70% ethanol for 2 min, and finally 2 min in sterile distilled water, before placing them on *Trichoderma*-specific agar medium as described (Chung & Hoitink, 1990), to monitor outgrowth.

RNA extraction and northern blot analysis

Total RNA was extracted by homogenizing frozen tissue in extraction buffer (0.35 M glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 $mL.g^{-1}$ plant tissue). The homogenates were extracted with phenol/chloroform/isoamylalcohol (25:24:1) and the RNA was precipitated with LiCl, as described previously (Sambrook *et al.*, 1989). For RNA gel blot analysis, 12.5 µg RNA was denatured using glyoxal and DMSO (Sambrook *et al.*, 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto a Hybond-N^+ membrane (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 gel blots were hybridized with *LOX2* (At3g45140)-specific probes that were labeled with α-^{32}P-dCTP by random primer labeling (Feinberg & Vogelstein, 1983). *LOX2* probes were generated through PCR on *A. thaliana* cDNA using gene-specific primers (5'-GCA TCC TCA TTT CCG CTA CAC CA-3' and 5'-TCC GCA CTT CAC TCC ACC ATC CT-3'). A gene-specific probe for 18S rRNA was used to check for equal loading.

ACKNOWLEDGEMENTS

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CHAPTER 4

Jasmonate-responsive genes that are primed during rhizobacteria-induced systemic resistance are enriched for a MYC2 binding motif

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ABSTRACT

Upon appropriate stimulation, plants develop an enhanced capacity to express infection-induced cellular defense responses, a phenomenon known as priming. Primed plants display an accelerated expression of basal defenses upon pathogen attack, resulting in an enhanced level of resistance against a broad spectrum of pathogens. Since this boost of basal defenses is expressed only after pathogen infection, priming is thought to entail ecological fitness benefits under disease pressure. Colonization of the roots of *Arabidopsis thaliana* by the beneficial rhizobacterial strain *Pseudomonas fluorescens* WCS417r primes the leaf tissue for enhanced pathogen- and insect-induced expression of jasmonate (JA)-responsive genes, resulting in an induced systemic resistance (ISR) that is effective against different types of pathogens and insect herbivores. Here we investigated the molecular mechanism of this rhizobacteria-induced priming response by following a whole-genome transcript profiling approach. Out of the 1879 genes that were responsive to exogenous application of methyl jasmonate (MeJA), 442 genes displayed an altered response to MeJA in plants treated with ISR-inducing WCS417r bacteria. Comparison of this ISR-primed, MeJA-responsive set of genes with previously published microarray data revealed that WCS417r-induced priming preferentially potentiates genes involved in JA-dependent defense responses that are activated upon pathogen or insect attack. *In silico* analysis of the first 1000 base pairs upstream of the 5′-untranslated region of the ISR-primed MeJA-responsive genes revealed that the motif CACATG is significantly over-represented in the promoters of these genes. Genes with a primed expression pattern in ISR-expressing plants after infection with the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 were similarly enriched for the CACATG motif in their promoters. This motif is a binding site for the basic helix-loop-helix transcription factor MYC2, which plays a central role in JA- and absicic acid-regulated signaling pathways. *MYC2* gene expression was consistently up-regulated in WCS417r-ISR-expressing plants. Moreover, *MYC2*-impaired mutants *jin1-1* and *jin1-2* were unable to mount WCS417r-ISR against *P. syringae* pv. *tomato* DC3000 and the downy mildew pathogen *Hyaloperonospora parasitica*. Together, our results pinpoint the transcription factor MYC2 as a potential regulator in priming for enhanced JA-responsive gene expression during rhizobacteria-mediated ISR.

INTRODUCTION

To survive, living organisms have evolved complex mechanisms to detect aggressors and to defend themselves. Generally, the key to effective defense is the timing and magnitude of the defense reactions that are triggered upon attack. In plants, genomics approaches have shown that the timing and amplitude of the transcriptional response of defense-related genes determines the effectiveness of the induced defense reaction and, therefore, the level of resistance to the attacker (Nimchuk *et al.*, 2003; Tao *et al.*, 2003; De Vos *et al.*, 2005). Induced plant defenses are regulated by a highly interconnected signaling network in which the plant hormones jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) play central roles (Pieterse & Van Loon, 1999; Glazebrook *et al.*, 2003; Pozo *et al.*, 2004; Mauch-Mani & Mauch, 2005). It is thought that spatio-temporal intensities of these alarm signals determine the specific nature of the defense response triggered as marked by the activation of an attacker-specific set of defense-related genes (Reymond & Farmer, 1998; Rojo *et al.*, 2003; De Vos *et al.*, 2005; Mur *et al.*, 2006). The outcome of the induced defense response seems to be finely tuned by regulatory elements in the promoters of the defense-related genes, resulting in quantitative and/or kinetic effects on the induced resistance response (Katagiri, 2004).

Plant cells can be sensitized to react faster and/or stronger to environmental stresses upon appropriate stimulation. This phenomenon is called priming and can be induced biologically by beneficial rhizobacteria (Verhagen *et al.*, 2004), mycorrhizal fungi (Pozo *et al.*, 2004), pathogens (Cameron *et al.*, 1999), and insect herbivores (Engelberth *et al.*, 2004; De Vos *et al.*, 2006b; Ton *et al.*, 2007), or chemically by exogenous application of low doses of SA (Mur *et al.*, 1996), its functional analogue benzothiadiazole (Katz *et al.*, 1998), JA (Kauss *et al.*, 1994) or ß-amino butyric acid (Jakab *et al.*, 2001; Cohen, 2002). In primed plants, defense responses are not activated directly by the priming agent, but are accelerated only following perception of biotic or abiotic stress signals, resulting in an enhanced level of resistance (Conrath *et al.*, 2002; 2006). Priming is a common feature of different types of induced resistance and may represent an important ecological adaptation to resist environmental stress (Pieterse & Dicke, 2007; Walters & Heil, 2007). By studying the costs and benefits of priming in *Arabidopsis*, it was recently shown that the fitness costs of priming are lower than those of constitutively activated defenses, such as expressed in the constitutive defense-expressing mutant *cpr1* (Van Hulten *et al.*, 2006). Hence, the costs of priming are outweighed by its benefits under conditions of relatively high disease pressure, suggesting that priming is a cost-efficient strategy of plants to cope with environmental stress.

Priming in beneficial plant-microbe interactions has been studied most extensively in the interaction of plants with plant growth-promoting rhizobacteria. These non-pathogenic rhizobacteria are abundantly present on the surface of plants roots, where they utilize nutrients that are exuded by the root cells (Bloemberg & Lugtenberg, 2001). Selected rhizobacterial strains are capable of reducing disease incidence in above-ground plant parts through a plant-mediated defense mechanism known as induced

systemic resistance (ISR) (Van Loon *et al.*, 1998). Rhizobacteria-mediated ISR has been demonstrated in many plant species, e.g. bean, carnation, cucumber, radish, tobacco, tomato and *Arabidopsis*, and is effective against a broad spectrum of plant pathogens including oomycetes, fungi, bacteria and viruses (Van Loon & Bakker, 2005; 2006), and even insects (Van Oosten, 2007). The first evidence of priming during ISR came from experiments with carnation (*Dianthus caryophyllus*) where upon inoculation with *Fusarium oxysporum* f.sp. *dianthi* phytoalexin levels increased to higher levels in ISR-expressing plants than in inoculated control 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). Upon challenge infection of the plants with the fungus, the walls of root cells were rapidly strengthened at the sites of attempted fungal penetration through appositions that contained callose and phenolic material, and fungal ingress was prevented effectively (Benhamou *et al.*, 1996).

In Arabidopsis, ISR triggered by *Pseudomonas fluorescens* WCS417r is regulated by a JA- and ET-dependent signaling pathway (Pieterse *et al.*, 1998). In contrast to pathogen-induced systemic acquired resistance (SAR), WCS417r-ISR is not associated with a direct activation of genes encoding PATHOGENESIS-RELATED (PR) proteins (Pieterse *et al.*, 1996). Analysis of the *Arabidopsis* transcriptome revealed that, locally in the roots, ISR-inducing WCS417r bacteria elicited a substantial change in the expression of almost 100 genes (Verhagen *et al.*, 2004; Léon-Kloosterziel *et al.*, 2005). However systemically, in the leaves, no consistent alteration in gene expression was observed, demonstrating that the onset of ISR is not associated with detectable changes in gene expression (Verhagen *et al.*, 2004). In addition, no alterations in the production of either JA or ET were detected in the leaves of induced plants expressing WCS417r-ISR, suggesting that the state of ISR relies on an enhanced sensitivity to these plant hormones rather than an increase in their production (Pieterse *et al.*, 2000).

Analysis of the transcriptome of ISR-expressing *Arabidopsis* leaves after challenge inoculation with the bacterial speck pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) revealed 81 genes with amplified expression patterns, indicating that the plants were primed to respond faster and/or more strongly to pathogen attack (Verhagen *et al.*, 2004). The majority of these primed genes was predicted to be regulated by JA and/or ET, confirming earlier findings that colonization of the roots by WCS417r primed *Arabidopsis* plants for potentiated expression of the JA- and/or ET-responsive genes *VSP2*, *PDF1.2*, and *HEL* (Van Wees *et al.*, 1999; Hase *et al.*, 2003). Other ISR-inducing rhizobacteria have also been demonstrated to enhance the plant's defense capacity by priming for potentiated expression of defense-related genes (e.g. De Meyer *et al.*, 1999a; Ahn *et al.*, 2002; Kim *et al.*, 2004; Tjamos *et al.*, 2005; Ahn *et al.*, 2007), which strongly suggests that priming is a common mechanisms in rhizobacteria-mediated ISR.

To gain more insight into how ISR-related priming is regulated, we followed a genome-wide expression profiling approach to identify JA-responsive *Arabidopsis* genes that show an augmented expression pattern in plants expressing WCS417r-ISR.

Subsequently, we performed an in silico promoter analysis to identify motifs that are potentially involved in the regulation of the priming response. Here, we provide evidence that the motif CACATG is significantly enriched in the 1-kb promoter regions of the JA-responsive ISR-primed genes. The CACATG motif has previously been demonstrated to be a binding site for the MYC-type helix-loop-helix transcription factor MYC2. We show that MYC2-impaired mutants *jin1-1* and *jin1-2* are blocked in their ability to express WCS417r-ISR, indicating that the MYC2 transcription factor is an essential regulator of WCS417r-ISR.

RESULTS

WCS417r-ISR primes the plant for enhanced MeJA-induced expression of *LOX2*

Previously, microarray analyses revealed that *Arabidopsis* plants expressing WCS417r-ISR are primed for accelerated expression of genes that are activated upon attack by *Pst* DC3000 (Verhagen *et al.*, 2004). The majority of these primed genes were predicted to be regulated by JA signaling, suggesting that WCS417-ISR is associated with an enhanced sensitivity of the induced tissues to JA that is produced upon pathogen attack. To test this hypothesis, the responsiveness of non-induced and WCS417r-ISR-expressing plants to exogenously applied MeJA was investigated. *Arabidopsis* Col-0 plants grown in soil with or without ISR-inducing WCS417r bacteria were treated with 50 µM MeJA and harvested 0, 1, 3, 6 and 12 hr later, after which the expression level of the JA-responsive marker gene *LOX2* (At3g45140) was determined by northern blot analysis. Figure 4.1 shows that in MeJA-treated control plants the *LOX2* transcript level started to rise already at 1 hr after MeJA treatment and reached a maximum at 6 hr. In WCS417r-ISR-expressing plants, *LOX2* transcript levels accumulated to higher levels at all time points tested. Moreover, at 12 hr after MeJA treatment *LOX2* mRNA levels were still high in WCS417r-ISR-expressing plants, whereas they had decreased to almost basal levels in MeJA-treated control plants. These results indicate that colonization of the roots by ISR-inducing WCS417r bacteria primes the leaf tissue for accelerated expression of JA-responsive genes such as *LOX2*.

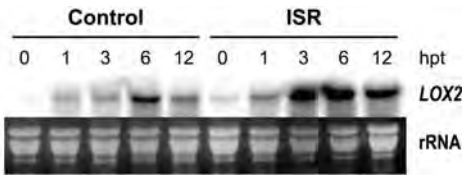

Figure 4.1. Priming for enhanced MeJA-responsive *LOX2* gene expression in ISR-expressing plants.

Northern blot analysis of *LOX2* transcript levels in the leaves of wild-type Col-0 plants grown in soil with or without ISR-inducing WCS417r bacteria. RNA was isolated at different time points after exogenous application of 50 µM MeJA. To check for equal loading, rRNA bands were stained with ethidium bromide. hpt, hours post treatment.

Identification of MeJA-responsive genes

To identify JA-responsive genes that, like *LOX2*, are primed during WCS417r-ISR, we analyzed the whole-genome transcript profile of control and WCS417r-ISR-expressing *Arabidopsis* Col-0 plants at different time points after MeJA treatment using whole-genome Affymetrix *Arabidopsis* ATH1 GeneChips (Redman *et al.*, 2004). Because the primed expression pattern of *LOX2* was clear within the first 6 h after MeJA treatment (Fig. 4.1), we performed the transcript profiling with leaf material that was harvested at 1, 3, and 6 h after application. Expressed genes were identified and expression values from each sample were normalized globally using GCOS. To obtain a robust set of MeJA-responsive genes, we applied the following selection criteria. Firstly, the expression level had to be significantly exceed background (P-flag generated by GCOS) and the hybridization intensity had to be >30 units at t=0 for down-regulated genes and in at least two of the later time points for the up-regulated genes (95% of all probe sets with a P-flag had averaged hybridization intensity levels of over 30 units). Secondly, the expression level in MeJA-treated leaves had to be at least twice that in control leaves (0 hr). To avoid false positives, we required the change to occur on at least two out of the three time points and in the same direction. Although the cut-off value of twofold is arbitrary, in combination with the additional timing and direction criteria this value has been demonstrated to yield a robust selection of gene sets (De Vos *et al.*, 2005). Probe sets corresponding to 1879 genes (955 up- and 924 down-regulated) met these criteria. These genes were considered MeJA-responsive genes and are listed in Supplemental Table 1. The list includes well-characterized JA-responsive genes that are commonly used as markers for JA- dependent responses, such as *PDF1.2* (At5g44420), *THI2.1* (AT1g72260), *VSP1* (At5g24780), *COR1* (At1g19670), *JR1* (At3g16470), *JR2* (At4g23600), and genes involved in the JA biosynthetic pathway, such as *AOS1* (At5g42650), *LOX2* (At3g45140), and *JMT* (At1g19640). To validate the GeneChip data obtained, we compared the selected MeJA-responsive genes with those identified in other transcript profiling studies in *Arabidopsis*. Sasaki and colleagues (2001) performed a similar time course study of the *Arabidopsis* response to MeJA treatment using a cDNA macroarray. Despite the different type of array used and experimental set-up, 80% of the MeJA-responsive genes described in their work also showed a more than twofold change in expression in our data (not shown).

Identification of ISR-primed MeJA-responsive genes

To identify genes that are primed to respond faster and/or with different amplitude to MeJA in WCS417r-ISR-expressing plants (so-called ISR-primed genes), we required the expression level of the MeJA-responsive genes listed in Supplemental Table 4.1 to be >1.5-fold different in MeJA-treated ISR-expressing plants versus MeJA-treated control plants. This priming cut-off value is based on previous quantitative expression data of genes that showed a robust primed expression pattern after pathogen attack (Verhagen *et al.*, 2004). A total of 442 genes met these selection conditions and are listed in

Supplemental Table 4.2. Although *LOX2* showed a clear ISR-primed expression pattern after MeJA treatment when northern blotting was used (Fig. 4.1), it is not listed in Supplemental Table 4.2. The reason is that *LOX2* transcript levels could not be reliably compared in the microarray analysis, because *LOX2* was among the eight MeJA-responsive genes whose probe sets were saturated (expression levels >5000 units) after hybridization with all probes from MeJA-treated plants.

To further verify the microarray data, we selected the ISR-primed MeJA-responsive gene *PYK10* (At3g09260) and analyzed its expression along with that of *LOX2* in an independent experiment. The response of *LOX2* and *PYK10* to MeJA treatment was assessed at 0, 1, 3, and 6 hr after treatment of the leaves of wild-type Col-0 and JA-insensitive *coi1-16* mutant plants that were grown in soil with or without ISR-inducing WCS417r bacteria. In a parallel set of plants, expression of ISR was verified using *Pst* DC3000 as a challenging pathogen. As expected, colonization of the roots by WCS417r bacteria induced systemic protection against *Pst* DC3000 in Col-0 (Fisher's LSD test; $P<0.001$) but not in *coi1-16* (Fisher's LSD test; $P<0.368$) (data not shown). Northern blot analysis and quantification of the hybridization signals with a Phosphor Imager showed that *LOX2* and *PYK10* mRNAs accumulated to high levels in MeJA treated Col-0 plants, whereas the transcript levels of both genes were much lower in MeJA-treated *coi1-16* plants (Fig. 4.2). Moreover, *LOX2* and *PYK10* mRNA levels were more than 1.5-fold higher in ISR-expressing Col-0 plants on at least 2 of the 3 time points tested, confirming the ISR-primed and MeJA-responsive expression pattern of these genes.

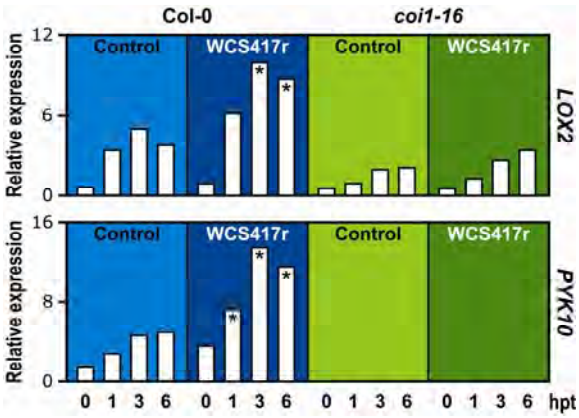

Figure 4.2. Priming for enhanced MeJA-responsive expression of *LOX2* and *PKY10* in WCS417r-treated Col-0 and *coi1-16* plants.

Quantitative northern blot analysis of *LOX2* and *PKY10* transcript levels in the leaves of wild-type Col-0 plants and JA-insensitive *coi1-16* mutant plants grown in soil with or without ISR-inducing WCS417r bacteria. RNA was isolated at different time points after exogenous application of 100 µM MeJA. Hybridization signals on the northern blots were quantified using a Phospho imager and normalized using *18S* rRNA signal intentities. Asterisks indicate that the ratio of the MeJA-induced expression level in WCS417r- over control-treated plants is at least 1.5-fold. hpt, hours post treatment.

Functional analysis of ISR-primed genes

MeJA-responsive genes were classified according to their functional categories derived from the Gene Ontology tool at The Arabidopsis Information Resource (TAIR)

(http://www.arabidopsis.org) (Rhee *et al.*, 2003). To evaluate the relevance of a given functional category, the percentage of differentially expressed genes belonging to the defined functional group was compared to the degree of representation of the respective functional category in the genome. Figure 4.3 shows the results of this comparison for the non-primed, MeJA-responsive genes as compared to the ISR-primed MeJA-responsive gene sets. The dominant functional categories that are overrepresented in the non-primed MeJA-responsive gene set are the categories "response to biotic and abiotic stress" (197.0%) and "response to biotic and abiotic stimulus" (218.6%). In the ISR-primed MeJA-responsive gene set, the overrepresentation of genes from these categories is even more pronounced (272.2% and 310.9%, respectively), suggesting that WCS417r-mediated priming predominantly affects genes that respond to biotic or abiotic stress.

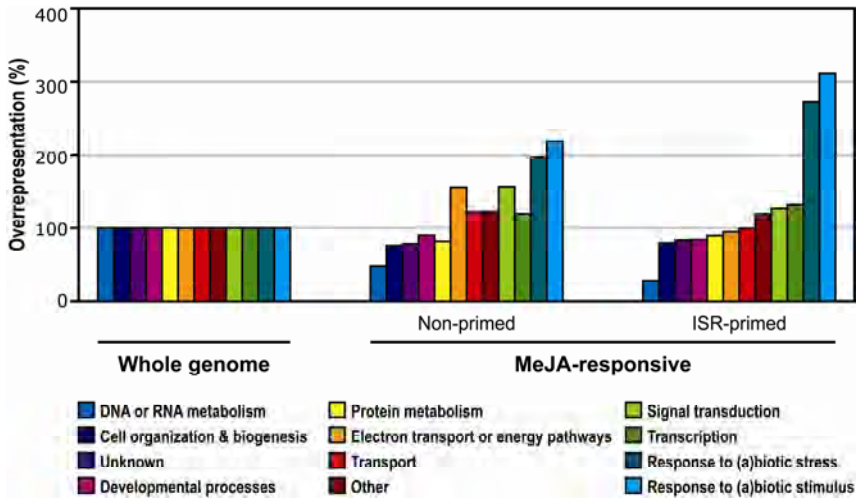

Figure 4.3. Functional analysis of ISR-primed and non-primed MeJA-responsive gene sets.

Depicted is the distribution of the ISR-primed and non-primed MeJA-responsive genes over the different functional categories. Classification in functional categories was performed essentially according to the Gene Ontology tool of TAIR. To visualize the degree of overrepresentation of the selected genes in the functional categories, the distribution of the ISR-primed and non-primed MeJA-responsive genes over the functional categories is presented relative to the distribution of all genes (whole genome) on the Affymetrix ATH1 array (set at 100% for each functional category).

To investigate whether the ISR-primed gene set is indeed enriched for genes that respond during biotic stress, we compared the sets of non-primed MeJA-responsive genes and ISR-primed MeJA-responsive genes from this study with gene sets that were previously demonstrated to be responsive to the bacterial leaf pathogen *Pst* DC3000, the necrotrophic fungus *Alternaria brassicicola*, tissue-chewing caterpillars of *Pieris rapae* (small cabbage white), or cell content-feeding *Frankliniella occidentalis* (Western flower

thrips) (De Vos *et al.*, 2005). These four attackers have in common that they all provoke an increase in JA biosynthesis and JA-responsive gene expression in *Arabidopsis* (De Vos *et al.*, 2005). Figure 4.4 shows that 35% of the non-primed MeJA-responsive genes were also responsive to one or more of the *Arabidopsis* attackers. Of the ISR-primed MeJA-responsive genes a notably larger proportion (51%) was responsive to one or more of the attackers. Moreover, while 7% of the non-primed MeJA-responsive genes was responsive to more than one attacker, this percentage was much higher for the ISR-primed MeJA-responsive genes (19%). Together, these results indicate that priming during WCS417r-ISR preferentially potentiates genes involved in JA-dependent defense responses that are activated upon pathogen or insect attack.

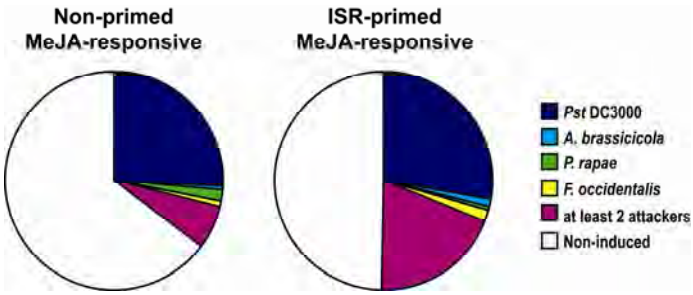

Figure 4.4. Responsiveness of ISR-primed and non-primed genes to JA-inducing pathogens and herbivores.

Proportion of non-primed and ISR-primed MeJA-responsive genes that are also responsive to the JA-inducing attackers *Pst* DC3000, *A. brassicicola*, *P. rapae* or *F. occidentalis*. Lists of attacker-responsive genes are taken from De Vos *et al.* (2005).

Selected ISR-primed MeJA-responsive genes also show a primed expression pattern upon pathogen attack

Because the ISR-primed genes were selected on the basis of their augmented expression after MeJA treatment, we anticipated that these genes would also show a primed expression pattern upon infection by a JA-inducing pathogen. Therefore, we analyzed the expression patterns of the ISR-primed MeJA-responsive genes *LOX2*, *PYK10*, *NMIN1*, and *WRKY54* in control- and WCS417r-treated Col-0 and *coi1-16* plants at 0, 3, 6 and 24 hr after challenge with *Pst* DC3000. Of the four selected genes, *LOX2* and *PYK10* are ISR-primed MeJA-responsive genes that show an accelerated up-regulation in ISR-expressing plants after MeJA treatment (Fig. 4.2; Supplemental Table 4.2), whereas MeJA-responsive expression of *NIMIN1* (At1g02450) and *WRKY54* (At2g40750) are suppressed in ISR-expressing plants after MeJA treatment (Supplemental Table 4.2). Expression of WCS417r-ISR against *Pst* DC3000 was confirmed for WCS417r-treated Col-0 plants, while *coi1-16* did not mount a significant level of protection against *Pst*

DC3000 (data not shown). *LOX2* and *PYK10* were induced in Col-0 after inoculation with *Pst* DC3000 (Fig. 4.5A) and showed a potentiated expression pattern in ISR-expressing Col-0 plants on at least 2 of the 3 time points tested. In contrast, in the *coi1-16* mutant, *Pst* DC3000-induced expression and WCS417r-mediated priming of the genes was fully abolished (*LOX2*) or strongly diminished for (*PYK10*). Also *NIMIN1* and *WRKY54* were induced in Col-0 upon inoculation with *Pst* DC3000 (Fig. 4.5B). However, in the ISR-expressing plants, expression of both genes showed a significantly lower level of induction after pathogen challenge, consistent with the WCS417r-mediated suppression of their expression in MeJA-treated plants. In *coi1-16* mutant plants, *Pst* DC3000-induced expression of *NIMIN1* and *WRKY54* was similar to that observed in Col-0 plants, indicating that the pathogen-induced expression of these genes is regulated in a JA-independent manner. Nevertheless, the WCS417r-mediated suppression of *Pst* DC3000-induced *NIMIN1* and *WRKY54* expression was blocked in *coi1-16* plants, indicating that the WCS417r-mediated priming of these genes was regulated in a JA-dependent manner. Collectively, these results confirm that the selected ISR-primed genes *LOX2, PYK10, NIMIN1,* and *WRKY54* show a similarly primed expression pattern in ISR-expressing plants upon treatment with MeJA or upon inoculation with the pathogen *Pst* DC3000.

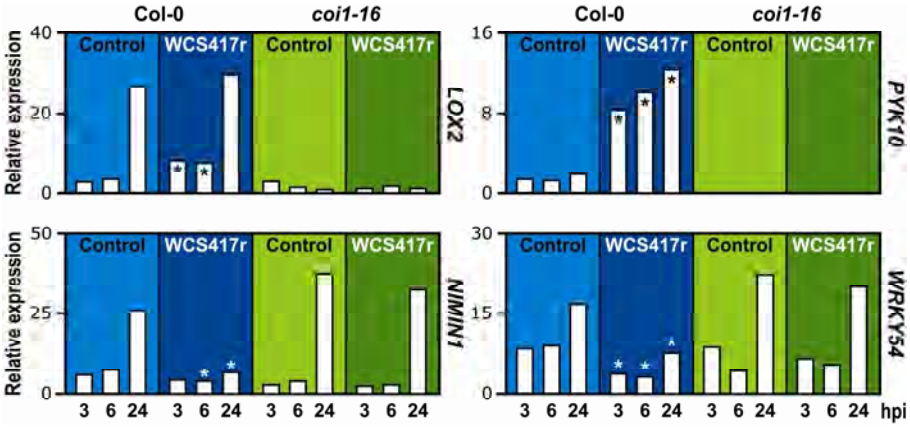

Figure 4.5. Primed expression patterns of *LOX2*, *PKY10*, *NIMIN1*, and *WRKY54* in WCS417r-treated Col-0 and *coi1-16* plants after inoculation with *Pst* DC3000.

Quantitative northern blot analysis of *LOX2, PKY10, NIMIN1,* and *WRKY54* transcript levels in the leaves of wild-type Col-0 plants and JA-insensitive *coi1-16* mutant plants grown in soil with or without ISR-inducing WCS417r bacteria. RNA was isolated at different time points after inoculation with *Pst* DC3000. Hybridization signals on the northern blots were quantified using a Phospho imager and normalized using *18S* rRNA signal intentities. Asterisks indicate that the ratio of the *Pst* DC3000-induced expression level in WCS417r- over control-treated plants is at least 1.5-fold. hpi, hours post inoculation.

Promoter analysis of ISR-primed genes

To gain further insight into the molecular mechanisms of priming for enhanced MeJA-responsive gene expression during WCS417r-ISR, we performed an *in silico* analysis of the promoter sequences of the selected ISR-primed genes to identify putative *cis*-regulatory elements. Functional *cis*-regulatory elements in plant promoters are typically found within the first 1 kilo base (kb) upstream of the ATG translation start site (Rombauts et al., 2003). Therefore, we scanned the 1-kb regions upstream the 5'-UTRs of the genes listed in Supplemental Tables 4.1 and 4.2, using the transcription factor binding site enrichment tool of the Athena database (http://www.bioinformatics2.wsu.edu/Athena) (O'Connor et al., 2005) to identify statistically over-represented *cis*-regulatory elements in ISR-primed versus non-primed MeJA-responsive genes. Table 4.1 shows the list of motifs that are enriched in the 1-kb promoter regions of the non-primed and ISR-primed MeJA-responsive genes. No unique motifs could be identified in the ISR-primed gene set, suggesting that the potentiated expression of these MeJA-responsive genes in ISR-expressing plants is caused by quantitative rather than qualitative differences in transcription factor activity.

Table 4.1. **P-values (hypergeometric distribution) of overrepresented motifs in promoters of non-primed MeJA-responsive and ISR-primed MeJA-responsive genes, compared to those of non-MeJA-responsive genes.**

Motif	Non-primed MeJA-responsive	ISR-primed MeJA-responsive
CArG-like (CWWWWWWWWG)	10^{-10}	10^{-8}
EveningElement (AAAATATCT)	10^{-7}	10^{-8}
W-box (TTGACY)	10^{-8}	10^{-8}
G-box related (CACATG)	10^{-11}	10^{-6}
ABRE-like (BACGTGKM)	10^{-10}	10^{-6}

To search for motifs that are specifically enriched in ISR-primed over non-primed genes, we statistically analyzed the frequency distribution of the identified transcription factor binding motifs in the promoters of the non-primed and the ISR-primed MeJA-responsive genes. To this end we used POBO, a promoter bootstrapping program that allows a three-way comparison between two clusters of co-regulated genes and the genomic background (Kankainen & Holm, 2004). Of the over-represented motifs in the MeJA-responsive genes, the G-box-related motif CACATG stood out as being the only one that was significantly more over-represented in the ISR-primed genes. Figure 4.6A shows that the promoters of the MeJA-responsive genes are significantly enriched in CACATG motifs in comparison to the genomic background. Yet, the CACATG motif is significantly more over-represented in the promoters of the ISR-primed MeJA-responsive genes as compared to the non-primed MeJA-responsive genes. If the CACATG motif is indeed associated with the primed expression pattern of JA-responsive genes, it could be

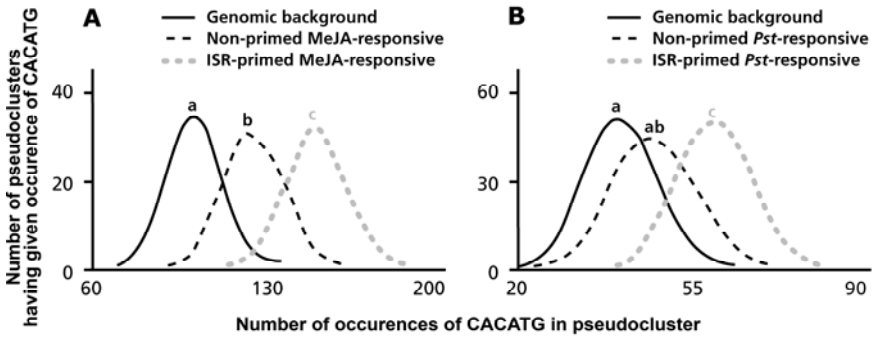

Figure 4.6. Frequency distribution of G-box-related CACATG motif in the promoter sequences of non-primed and ISR-primed Arabidopsis genes.

Occurrence of CACATG motifs was quantified in the 1-kb sequences upstream of the 5'-UTR of the non-primed and ISR-primed MeJA-responsive genes (A) **and non-primed and ISR-primed *Pst* DC3000-responsive genes** (B) **using POBO bootstrapping analysis (Kankainen & Holm, 2004). The non-primed and ISR-primed genes were compared to randomly selected promoter sequences from the *Arabidopsis* genome. Non-primed and ISR-primed *Pst* DC3000-responsive genes are taken from Verhagen *et al.* (2004). Different letters indicate statistically signicant differences in the occurrence of the *cis*-acting element (χ2 test; α=0.05).**

expected that the promoters of the previously identified ISR-primed *Pst* DC3000-responsive genes (Verhagen et al., 2004) are also enriched for this transcription factor binding site. Indeed, POBO analysis of the 1-kb promoter region of the 81 ISR-primed *Pst* DC3000- responsive genes from this study revealed that the CACATG motif is significantly over-represented in these genes (Fig. 4.6B). Together, these results suggest a functional role for this motif in the priming for enhanced JA-responsive gene expression during WCS417r-ISR.

The MYC2 transcription factor is required for WCS417r-ISR

The CACATG motif was previously identified as a binding site for the MYC2 transcription factor in the promoter of the ABA- and drought-responsive gene *rd22* (*responsive to dehydration 22*) (Abe *et al.*, 1997). The transcription factor MYC2 also plays an important role in the regulation of JA-responsive gene expression and defense against pathogen and insect attack (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Lorenzo & Solano, 2005; Dombrecht *et al.*, 2007). To investigate whether MYC2 is required for the expression of WCS417r-ISR, we tested the ability of the MYC2-impaired mutants *jin1-1* and *jin1-2* (Berger *et al.*, 1996; Lorenzo *et al.*, 2004) to express WCS417r-ISR against the pathogens *Pst* DC3000 and *H. parasitica*. Figure 4.7A shows that WCS417r-ISR resulted in a significant level of protection of Col-0 plants against *Pst* DC3000. However, mutant *jin1-1* and *jin1-2* failed to develop ISR against this pathogen. Similarly, *jin1-2* failed to develop ISR against *H. parasitica* (Fig. 4.7B). Moreover, priming for enhanced deposition

of callose-containing papillae at sites of attempted penetration of *H. parasitica*, a typical reaction of WCS417r-ISR-expressing Col-0 plants (Chapter 2), was significantly reduced in WCS417r-treated *jin1-2* plants (Fig. 4.7C).

To investigate whether WCS417r-ISR is associated with an increase in the level of *MYC2* mRNA, we analyzed *MYC2* transcript levels in non-induced control and WCS417r-ISR-expressing Col-0 plants in five independent experiments in which significant levels of ISR were detected (data not shown). Q-PCR analysis revealed that *MYC2* transcript levels were consistently raised 1.6- to 3.5-fold in WCS417r-ISR-expressing plants (Fig. 4.8). In *npr1-1* mutant plants that are unable to mount an ISR response (Pieterse *et al.*, 1998), the level of *MYC2* mRNA was not elevated upon colonization of the roots by WCS417r (Fig. 4.8B). Thus colonization of the roots by ISR-inducing WCS417r bacteria results in a moderate, but consistently enhanced expression of the transcription factor gene *MYC2*. Together, these results demonstrate that MYC2 is required for the expression of WCS417r-ISR.

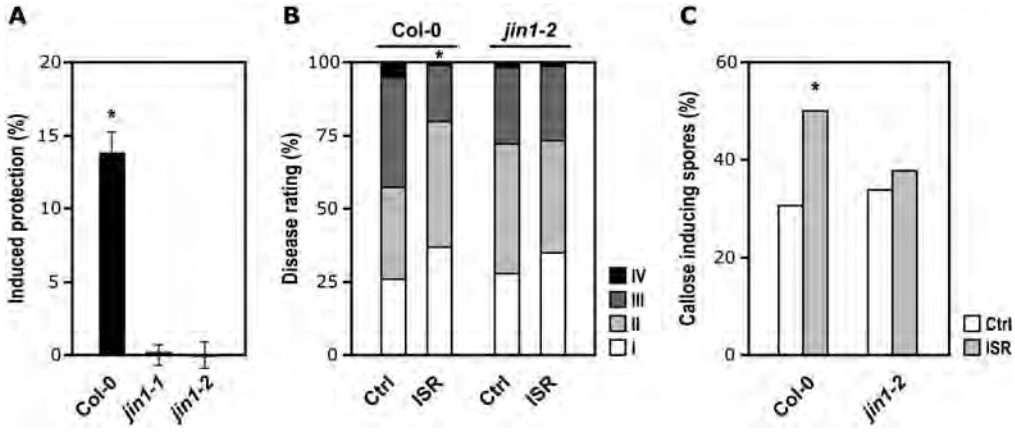

Figure 4.7. WCS417r-ISR against *Pst* DC3000 and *H. parasitica* is blocked in *myc2/jin1* mutants.

(A) **Levels of induced protection against *Pst* DC3000 in Col-0 and *MYC2* mutants *jin1-1* and *jin1-2*. ISR was induced by growing the plants for three weeks in soil containing ISR-inducing WCS417r bacteria. Five-week-old plants were challenge inoculated with a bacterial suspension of virulent *Pst* DC3000. Four days after challenge inoculation, the percentage of diseased leaves was assessed and the level of induced protection calculated on the basis of the reduction in disease symptoms relative to challenged, non-induced plants. Asterisks indicate statistically significant differences compared to non-induced control plants (Students *t*-test, α=0.05, n=20).** (B) **Quantification of ISR against *H. parasitica* in Col-0 and *jin1-2*. ISR was induced by growing the plants in soil containing ISR-inducing WCS417r bacteria. Plants were challenge inoculated with *H. parasitica* when three weeks old. Disease severity was determined seven days after challenge. Disease ratings are expressed as the percentage of leaves (*n* = ~250) in disease-severity classes: I, no sporulation; II, trailing necrosis; III, <50% of the leaf area covered with sporangia; IV, >50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse. Asterisks indicate statistically significantly different distributions of the disease severity classes compared with the non-induced control treatments (χ2 test, α=0.05).** (C) **Induced resistance against *H. parasitica* is associated with enhanced deposition of callose-containing papillae at sites of attempted penetration, resulting in a reduction of the number of spores that successfully penetrate into *Arabidopsis* leaves. Two days after challenge with *H. parasitica*, the the proportion of spores that induced the formation of callose-containing papillae around the infection site was quantified in leaves of Col-0 and *jin1-2* plants (χ2 test, α=0.05).**

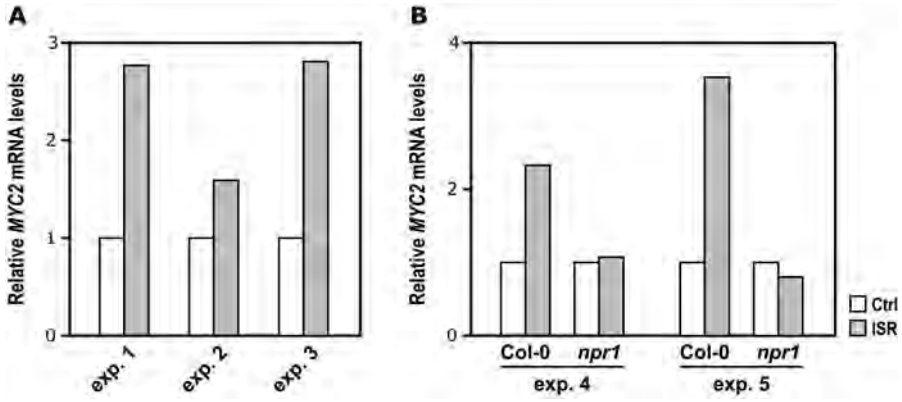

Figure 4.8. WCS417r-induced *MYC2* expression.

(A) **and** (B) **Q-PCR analysis of *MYC2* transcript levels in the leaves of five-week-old Col-0 and ISR-impaired *npr1* plants of which the roots were treated or not with ISR-inducing WCS417r bacteria. *MYC2* transcript levels in un-induced control plants were set at 1.**

DISCUSSION

Induced resistance is often associated with the production of defensive compounds such as PR proteins with anti-microbial activity (Van Loon *et al.*, 2006b), proteinase inhibitors that affect insect feeding (Howe, 2004), or volatiles that attract parasitoids and predators of the herbivores that feed on the plant (Van Poecke & Dicke, 2004). However, the enhanced defensive capacity in induced plants often can not be attributed to direct activation of defenses. In these cases, broad-spectrum protection of induced plants seem to be based on a faster and stronger activation of basal defense mechanisms upon exposure to either microbial pathogens or herbivorous insects. It is therefore hypothesized that the broad-spectrum characteristic of induced resistance is largely based on priming of the tissue to react more effectively to a stress condition, rather than on direct activation of defenses (Conrath *et al.*, 2002; Conrath *et al.*, 2006).

WCS417r-ISR in *Arabidopsis* emerged as a good model system to study the molecular mechanisms underlying priming for enhanced defense. WCS417r-ISR is not associated with direct activation of defense-related genes (Pieterse *et al.*, 1996), but rather on priming for enhanced attacker-induced expression of JA- and ET-responsive genes (Van Wees *et al.*, 1999; Hase *et al.*, 2003; Verhagen *et al.*, 2004; Van Oosten, 2007). Similarly, ISR-inducing *Pseudomonas putida* LSW17S was demonstrated to prime JA- and ET-dependent defense responses of *Arabidopsis* (Ahn *et al.*, 2007). In several other interactions between plants and plant growth-promoting rhizobacteria, increased resistance arises from a potentiated expression of defense-related genes (Benhamou *et al.*, 1996; De Meyer *et al.*, 1999a; Ahn *et al.*, 2002; Kim *et al.*, 2004; Tjamos *et al.*, 2005), suggesting that priming for enhanced defense is a common mechanism by which ISR-inducing rhizobacteria confer broad-spectrum resistance.

In this study, we identified a large group of MeJA-responsive genes of which the expression kinetics was changed in WCS417r-ISR-expressing plants. We demonstrated that these ISR-primed MeJA-responsive genes are enriched for genes with a function in the response of the plant to biotic or abiotic stress (Fig. 4.3 and 4.4). This suggests that WCS417r-induced priming selectively affects the expression of genes with a putative function in the adaptive response of the plant to changes in its environment. *In silico* analysis of the promoters of the ISR-primed MeJA-responsive genes revealed that, when compared to the non-primed MeJA-responsive genes, the primed genes contain a significantly larger number of the G-box-related motif CACATG (Fig. 4.6). Not only the ISR-primed MeJA-responsive genes were enriched for this motif, also the previously identified ISR-primed *Pst* DC3000-responsive genes (Verhagen *et al.*, 2004) contained a significantly larger number of CACATG motifs in their promoters (Fig. 4.6), suggesting a role for this motif in WCS417r-mediated priming for enhanced JA-responsive gene expression. The G-box-related motif CACATG was previously shown to function as a binding site for the basic helix-loop-helix (bHLH) domain–containing transcription factor MYC2 (Abe *et al.*, 1997; De Pater *et al.*, 1997). Besides binding to G-box-related sequences such as CACATG, MYC2 has been demonstrated to preferentially bind to the G-box sequence CACGTG (Dombrecht *et al.*, 2007). The MYC2 locus was first identified in a mutant screen for reduced sensitivity to JA (Berger *et al.*, 1996) and is allelic to *JASMONATE-INSENSITIVE 1* (*JAI1/JIN1*) (Lorenzo *et al.*, 2004). In several studies, MYC2 has been demonstrated to play an important role in the regulation of JA- and ABA-responsive genes (Abe *et al.*, 1997; Abe *et al.*, 2003; Anderson *et al.*, 2004; Boter *et al.*, 2004; Lorenzo *et al.*, 2004; Lorenzo & Solano, 2005). On the one hand, MYC2 acts as a negative regulator of JA-responsive genes such as *PDF1.2* and *HEL*, both of which are associated with defense against pathogens (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004). On the other hand, MYC2 functions as a positive regulator of JA-responsive genes such as *LOX2* and *VSP2* (*VEGETATIVE STORAGE PROTEIN 2*), which are associated with the wound response (Boter *et al.*, 2004; Lorenzo *et al.*, 2004). Moreover, MYC2 modulates the expression of JA-responsive transcription factor genes, thereby indirectly affecting the expression of a large number of downstream JA-responsive genes (Dombrecht *et al.*, 2007). Mutations in the *myc2/jin1* gene affect the level of resistance against pathogens *Botrytis cinerea, Plectosphaerella cucumerina, Fusarium oxysporum,* and *P. syringae* (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Nickstadt *et al.*, 2004; Laurie-Berry *et al.*, 2006) and the insect herbivore *Helicoverpa armigera* (Dombrecht *et al.*, 2007), highlighting the important regulatory function of this transcription factor in plant defense.

If MYC2 is important for WCS417r-mediated priming for enhanced JA-responsive gene expression, then one would expect that mutations in the *MYC2* gene affect the ability to develop ISR. Indeed, *MYC2* mutants *jin1-1* and *jin1-2* were blocked in their ability to mount WCS417r-ISR against *Pst* DC3000 (Fig. 4.6). Moreover, ISR-expressing Col-0 plants accumulated higher levels of *MYC2* mRNA, whereas the ISR-impaired mutant *npr1* did not (Fig. 4.8), suggesting that elevated *MYC2* mRNA levels are involved

in conferring enhanced resistance. This is corroborated by the observation that over-expression of *MYC2* does not lead to increased expression of JA- or ABA-responsive genes, but rather enhances the sensitivity of these plants to JA and ABA (Abe *et al.*, 2003; Boter *et al.*, 2004).

In conclusion, our results suggest the following model for WCS417r-induced priming of JA-dependent defenses during ISR: Colonization of the roots by ISR-inducing WCS417r bacteria leads to a systemic induction of the expression of the *MYC2* transcription factor gene. In the absence of a JA-inducing attacker, this induced *MYC2*-expression does not result in enhanced defense-related gene expression. Upon attack by a JA-inducing pathogen, WCS417r-induced plants are sensitized to accelerate the expression of MYC2-regulated JA-responsive genes, resulting in enhanced resistance against the JA-inducing attacker encountered.

Priming of defense responses can allow the plant to react faster, and therefore, more effectively to the invader encountered. In contrast to constitutive activation of defense mechanisms, priming confers flexibility to adapt the response to a specific challenge, leading to a less costly and broad-spectrum resistance. This seems to be the mechanism operating in rhizobacteria-induced ISR. The information derived from this research constitutes an important step forward in our knowledge on plant defense regulation and it opens new possibilities for the development of efficient biocontrol strategies. Similarly to prime-boost strategies, effective in eliciting protective cellular immunity to a variety of pathogens in humans, the idea of "boosting" immune responses in plants appears as a powerful strategy for environmentally friendly and durable crop protection.

EXPERIMENTAL PROCEDURES

Growth conditions of rhizobacteria and plants

Non-pathogenic *Pseudomonas fluorescens* WCS417r bacteria were used for the induction of ISR. WCS417r was grown for 24 hours at 28°C on King's medium B agar plates (King *et al.*, 1954) containing the appropriate antibiotics as described previously (Pieterse *et al.*, 1996). Bacteria were collected and resuspended in 10 mM $MgSO_4$ to a density of 10^9 cfu.mL^{-1} (OD_{660}=1.0) before being mixed through soil.

Seeds of wild-type *Arabidopsis thaliana* accession Col-0 and mutants *coi1-16* (Ellis & Turner, 2002), *jin1-1*, and *jin1-2* (Lorenzo *et al.*, 2004) were sown in quartz sand. Two-week-old seedlings were transferred to 60-ml pots containing a sand/potting soil mixture that had been autoclaved twice for 20 min with a 24-h interval. Before transfer of the seedlings, a suspension of ISR-inducing WCS417r bacteria (10^9 cfu.mL^{-1}) was mixed through the soil to a final density of $5x10^7$ cfu.g^{-1}, as described previously (Pieterse *et al.*, 1996). Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. Plants were cultivated in a growth chamber with a nine-hr day (200 μE.m^{-2}.sec^{-1} at 24ºC) and a 15-hr night (20ºC) cycle at 70% relative humidity. Plants were watered twice a week

with water and once a week with modified half-strength Hoagland nutrient solution (Hoagland & Arnon, 1938).

MeJA treatment

Induction treatment with methyl jasmonate (MeJA) was performed by dipping five-week-old Col-0 plants in an aqueous solution containing 50 µM MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands) and 0.015% of the surfactant Silwet L-77 (Van Meeuwen Chemicals B.V., Weesp, the Netherlands), as described previously (Pieterse *et al.*, 1998). Leaf rosettes were harvested at 0, 1, 3, 6 and 12 hr after induction treatment and immediately frozen in liquid nitrogen. Plants harvested 12-hr after treatment were kept under continuous light to avoid disruption of the time course by a dark period.

Pathogen inoculations and ISR bioassays

The virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Whalen *et al.*, 1991) and the oomycete *Hyaloperonospora parasitica* strain WACO9 (Van Hulten *et al.*, 2006) were used for challenge inoculation. *Pst* DC3000 was grown overnight in liquid King's medium B at 28°C. Bacterial cells were collected by centrifugation and resuspended to a final density of $2.5x10^7$ cfu.mL^{-1} in 10 mM $MgSO_4$ containing 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands). Five-week-old plants were placed at 100% relative humidity one day prior to challenge inoculation. Plants were inoculated by dipping the leaves for two seconds in *Pst* DC3000 suspension. Plants were harvested at 0, 3, 6, 24 and 48 h after challenge inoculation. To confirm expression of ISR in WCS417r-treated plants, ISR bioassays were performed as described previously (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997), using a subset of plants grown in parallel with the plants for the chemical treatment. Four days after challenge inoculation, disease severity was assessed by determining the percentage of diseased leaves per plant. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. The disease index was calculated for each plant (n=20), based on the percentage of diseased leaves.

H. parasitica bioassays were performed as described previously (Van Hulten *et al.*, 2006). Three-week-old plants were misted with a *H. parasitica* WACO9 spore suspension containing $7.5x10^4$ sporangiospores mL^{-1}. Inoculated plants were maintained at 17°C and 100% relative humidity for 24 hours. Subsequently, humidity was lowered to 70% to reduce effects on plant development and to lower the chance of secondary infections by opportunistic pathogens. Seven days after challenge inoculation humidity was raised again to 100% to induce sporulation. Disease symptoms were scored for ~250 leaves per treatment at nine days after inoculation. Disease rating was expressed on the basis of symptom severity and pathogen sporulation on each leaf: I, no sporulation; II, trailing necrosis; III, < 50% of the leaf area covered by sporangia; IV, >

50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse. In addition, 15 leaves per treatment were stained for quantification of callose deposition.

Callose staining

Quantification of callose deposition was performed as described by Ton *et al.* (2005). Leaves were collected two days after *H. parasitica* inoculation and incubated overnight in 96% ethanol. Destained leaves were washed in 0.07 M phosphate buffer, pH 9, incubated for 15 min in 0.07 M phosphate buffer containing 0.005% Calcofluor (fluorescent brightener; Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and 0.01% aniline-blue (water blue; Merck, Darmstadt, Germany), and then washed in 0.07 M phosphate buffer containing only 0.01% aniline-blue to remove excess Calcofluor. Observations were performed with an epifluorescence microscope with UV filter (band pass 340 to 380 nm, long-path 425 nm). Callose depositions were quantified by determining the percentage of callose-inducing spores per infected leaf; representative views of the stained leaves were photographed.

RNA gel-blot analysis

Total RNA was obtained by phenol/chloroform extraction and LiCl precipitation, as described by (Sambrook *et al.*, 1989). For RNA gel-blot analysis, 15 µg of total RNA were denatured in formamide, electrophoretically separated on 1.2% formaldehyde agarose gels and blotted onto Hybond-N^+ membranes (Amersham, `s-Hertogenbosch, the Netherlands) by capillary transfer. Equal loading was visualized by ethidium bromide staining of rRNA. Templates for the preparation of gene-specific probes were prepared by PCR with primers based on the annotated gene sequences. DNA probes were labelled with α-^{32}P-dCTP by random primer extension and hybridizations were carried out overnight at 42^{o}C using Ultrahyb (Ambion, Huntingdon, UK). Blots were exposed for autoradiography and signals quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, the Netherlands) with Quantity One software (BioRad, Veenendaal, the Netherlands).

Quantitative real-time PCR (Q-PCR)

Gene expression analysis by Q-PCR was performed basically as described previously (De Vos *et al.*, 2005). To check for contamination with genomic DNA, a PCR with primers designed for *EIL2* (At5g21120; $EIL2_F$ 5′- TCT CGT GAG ACG GTC TAG AAG TT-3′ and $EIL2_R$ 5′-ATG AAA CCT AAT CTT CTC CAT TGC-3′) was carried out. Subsequently, DNA-free total RNA was converted into cDNA using oligo-dT_{20} primers (Invitrogen, Breda, the Netherlands), 10 mM dNTPs, and SuperScript™ III Reverse Transcriptase (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Efficiency of cDNA

synthesis was assessed by Q-PCR, using primers of the constitutively expressed gene *UBI10* (At4g05320; $UBI10_F$ 5′-AAA GAG ATA ACA GGA ACG GAA ACA TAG T-3′ and $UBI10_R$ 5′-GGC CTT GTA TAA TCC CTG ATG AAT AAG-3′). Based on the results, cDNA of each sample was diluted to obtain a *UBI10* C_T (threshold cycle) value of 18 ± 0.5. Gene-specific primers for *MYC2* (At1g32640; MYC_F 5′-GAT GAG GAG GTG ACG GAT ACG GAA-3′ and $MYC2_R$ 5′-CGC TTT ACC AGC TAA TCC CGC A-3′) were designed previously by Czechowski *et al*. (2004). Q-PCR reactions were performed in a volume of 20 µl, containing cDNA, 0.5 µL of each of the two gene-specific primers (10 pmol.µL^{-1}), and 10 µL of 2x IQ SYBR® Green Supermix reagent. The following PCR program was used for all PCR reactions: 95°C for 3 min; 40 cycles of 95°C for 30 sec, 59.5°C for 30 sec, and 72°C for 30 sec. C_T (threshold cycle) values were calculated using Optical System Software, version 1.0 for MyIQ™ (Bio-Rad, Veenendaal, the Netherlands). C_T values were normalized for differences in dsDNA synthesis using the *UBI10* C_T values. Normalized transcript levels of each gene were compared in ISR-expressing plants and non-induced controls and the relative levels of transcription were calculated by using the $2^{\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Melting curves were recorded after cycle 40 by heating from 55°C to 95°C with a ramp speed of 1.9°C min^{-1}.

Sample preparation, microarray data collection, and transcript profiling

For isolation of RNA, whole leaf rosettes were harvested at 0, 1, 3 and 6 h after MeJA treatment and immediately frozen in liquid nitrogen. RNA was prepared from eight biological replicates that were pooled to reduce noise arising from biological variation. Total RNA was prepared as described above and cleaned using RNeasy Plant Mini Kit columns (Qiagen Benelux BV, Venlo, the Netherlands). RNA samples were analyzed for quality by capillary electrophoresis using an Agilent 2100 Bioanalyzer system. cRNA probe synthesis, hybridization to a GeneChip, and collection of data from the hybridized GeneChip were performed as described previously (De Vos *et al.*, 2005). Hybridizations with labeled cRNAs were conducted with *Arabidopsis* ATH1 full-genome GeneChips (Affymetrix, Santa Clara, USA), containing a total number of 22,810 probe sets representing approximately 23,750 *Arabidopsis* genes (Redman *et al.*, 2004). Probe preparations and GeneChip hybridizations were carried out by ServiceXS (Leiden, the Netherlands) and the Affymetrix service station of Leiden University Medical Centre (LUMC) where they passed all internal quality checks.

After hybridization, GeneChips were analyzed by using the GeneChip Operating Software (GCOS) (Affymetrix, Santa Clara, USA) and GeneSpring 6.1 (Silicon Genetics, Redwood, CA, USA), as previously described (De Vos *et al.*, 2005). The P-values from the Pearson correlation tests for GeneChips that were hybridized with probes from samples from the same time point ranged between 0.979 and 0.992. This is in good agreement with the high correlation coefficients previously reported for independent biological samples (Redman *et al.*, 2004) and indicates that the GeneChip hybridizations and microarray data collection were performed in a technically sound manner.

Expressed genes were identified using GCOS, which uses statistical criteria to generate a 'present' (P-flag) or 'absent' (A-flag) call for genes represented by each probe set on the array. The average number of detectable genes with 'present' call was 14,296 (62.7%), which is in good agreement with the 60% previously reported by Redman *et al.* (2004). Expression values from each sample were normalized globally by GCOS using a target intensity of 200 for global scaling. Genes with accurately detectable transcript levels were defined by probe sets showing averaged expression levels greater than 30 (95% of all probe sets with a 'present' call had a signal intensity above 30). Probe sets showing an expression value of <30 were adjusted to 30 to exclude false positives.

Regulatory motif analysis of promoter regions

For the analysis of the frequency distribution of *cis*-acting elements in the promoters of selected groups of genes, 1 kb of genomic DNA sequences upstream from the inferred translational start sites were downloaded from TAIR (http://www.arabidopsis.org /tools/bulk/sequences/index.html). To identify significantly over-represented putative regulatory sequences, the 1-kb regions of genes belonging to the selected groups were analysed using the Athena database (http://www.bioinformatics2.wsu.edu/Athena) (O'Connor *et al.*, 2005). Once putative regulatory sequences were selected, the statistical significance of their differential distribution in selected clusters was verified using POBO, a promoter bootstrapping program that allows a three-way comparison between two clusters and the background (Kankainen & Holm, 2004). The parameters used were: Number of pseudoclusters: 1000, number of promoters in the pseudo-clusters: number of promoters in the smallest group to be compared.

SUPPLEMENTARY MATERIAL

Supplementary Table 4.1. MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected MeJA-responsive genes.

Supplementary Table 4.2. MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected ISR-primed MeJA-responsive genes.

All supplementary materials can be downloaded from:
http://www.bio.uu.nl/~fytopath/GeneChip_data.htm

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CHAPTER 5

Priming for defense by rhizobacteria and ß-amino butyric acid: differential effects on callose deposition and transcription factor gene expression

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ABSTRACT

Arabidopsis develops systemically induced resistance to *Hyaloperonospora parasitica* and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) upon colonization of the roots by the non-pathogenic rhizobacterial strain *Pseudomonas fluorescens* WCS417r or by treatment with the non-protein acid ß-amino butyric acid (BABA). Both WCS417r-induced systemic resistance (WCS417r-ISR) and BABA-induced resistance (BABA-IR) are based on priming of defense responses. As opposed to direct activation of defense, priming is characterized by an enhanced capacity to pathogen attack. In this comparative study we investigated the role of two cellular defense responses in WCS417r- and BABA-induced priming: 1) the formation of callose-rich papillae at the site of attempted pathogen entry, and 2) the activation of transcription factor (TF) genes. Both WCS417r-ISR and BABA-IR were associated with an enhanced capacity to form callose-rich papillae at the site of *H. parasitica* spore penetration, resulting in an elevated level of resistance against this pathogen. Mutant analysis revealed that both WCS417r- and BABA-induced priming for enhanced papillae deposition require IBS2 (SAC1b) and IBS3 (ABA1/NPQ2), indicating that ISR and BABA-IR share signaling components in the regulation of this priming response. In contrast, both WCS417r-ISR and BABA-IR appeared to function independently of IBS2 and IBS3 against *Pst* DC3000, indicating that against this pathogen WCS417r- and BABA-induced priming for enhanced defense are not mediated by enhanced callose deposition. Both WCS417r-ISR and BABA-IR against *Pst* DC3000 are NPR1-dependent. However, whereas WCS417r-ISR against this pathogen is regulated by the TF MYB72, BABA-IR is not. Moreover, WCS417r-ISR was associated with priming for enhanced expression of the jasmonate (JA)-inducible gene *LOX2*, whereas BABA-IR was related to priming of the SA/NPR1-inducible gene *PR-1*. To investigate the role of TFs in this differential priming response, we analyzed the expression of all potential *Arabidopsis* TF genes upon induction of the primed state by WCS417r and BABA using a robotized quantitative reverse-transcriptase PCR approach. Both WCS417r and BABA treatments altered the expression of a large number of largely non-overlapping TF genes in the primed leaves. Notably, BABA specifically activated the transcription of 21 WRKY TF genes, 20 of which were dependent on NPR1. Promoter analysis of selected priming-associated TF genes revealed overrepresentation of specific *cis*-acting elements that support their involvement in plant defense. Collectively, we demonstrate that WCS417r- and BABA-induced priming is at least partly based on a similar IBS2- and IBS3-dependent enhanced capacity to form callose-rich papillae, and that both priming responses are marked by differential expression of divergent sets of TF genes. We hypothesize that increased levels of the corresponding TFs in the primed cells result in an enhanced capacity to activate pathogen-responsive genes upon challenge inoculation, leading to an increased level of resistance.

INTRODUCTION

Plants have evolved sophisticated mechanisms to defend themselves against microbial pathogens. Apart from constitutive barriers, plants rely on a large spectrum of inducible defense mechanisms. Well-characterized examples of such pathogen-inducible defenses are the production of anti-microbial compounds, such as phytoalexins and PATHOGENESIS-RELATED (PR) proteins, as well as the formation of cell wall appositions at sites of fungal or oomycetous attack (Hammerschmidt, 1999; Van Loon & Van Strien, 1999; Ton & Mauch-Mani, 2004). The plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) are important regulators of these stress-inducible defenses (Glazebrook, 2005; Mauch-Mani & Mauch, 2005; Van Loon *et al.*, 2006a; Seki *et al.*, 2007). Together, constitutive and pathogen-inducible defense mechanisms constitute the plant's basal defense, comparable to innate immunity in animals (Ausubel, 2005).

As a result of the evolutionary arms race between plants and their attackers, many microbial pathogens have evolved the ability to circumvent or suppress basal defense. Consequently, plants have adapted to express additional layers of defense that allow them to detect specific invaders at an early stage of infection. The extensively studied mechanisms underlying *R*- (*RESISTANCE*-) gene-dependent resistance, also known as effector-triggered immunity (ETI), form the key to this superimposed layer of defense (McDowell & Woffenden, 2003; Jones & Dangl, 2006).

In addition to basal resistance and *R*-gene dependent resistance, many plants have the ability to acquire an enhanced defensive capacity upon perception of selected biotic or abiotic stimuli, such as pathogens, selective root-colonizing bacteria, or specific chemicals (Van Loon, 2000). This so-called induced resistance does not necessarily require direct activation of defense mechanisms, but can also result from a sensitization of the tissue to express basal defense mechanisms faster and more strongly upon subsequent pathogen attack. The latter phenomenon is called priming (Conrath *et al.*, 2006). As demonstrated recently, priming of the plant's innate immune system yields broad-spectrum resistance with minimal reductions in plant growth and seed set (Van Hulten *et al.*, 2006). Hence, priming constitutes a cost-efficient resistance strategy that increases the plant's ability to respond to environmental stress.

The classic form of induced resistance develops upon limited infection by a pathogen, and results in a systemic acquired resistance (SAR) that protects against various types of pathogens (Ryals *et al.*, 1996; Durrant & Dong, 2004). The signaling pathway controlling SAR depends on the accumulation of endogenous SA (Gaffney *et al.*, 1993) and on the presence of the defense regulatory protein NPR1 (NONEXPRESSOR OF PR-GENES 1) (Cao *et al.*, 1994). NPR1 functions in SA-dependent basal resistance as well as in SAR, and has been shown to control the expression of many stress-related genes, including those encoding PR-proteins (Van Loon *et al.*, 2006b) and proteins involved in the secretory pathway (Wang *et al.*, 2005).

Colonization of plant roots by the non-pathogenic rhizobacterial strain *Pseudomonas fluorescens* WCS417r also triggers a systemic resistance against a wide range of pathogens, including the bacterial speck pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and the downy mildew-causing oomycete *Hyaloperonospora parasitica* (Pieterse *et al.*, 1996; Ton *et al.*, 2002b). In contrast to pathogen-induced SAR, this so-called rhizobacteria-induced systemic resistance (ISR) functions independently of SA, but requires components of the JA and ET response pathways (Pieterse *et al.*, 1998). Unlike pathogen-induced SAR, ISR elicited by WCS417r (WCS417r-ISR) is not accompanied by the direct activation of *PR* genes (Pieterse *et al.*, 1996). Instead, ISR-expressing plants are primed for enhanced expression of predominantly JA- and ET-responsive genes after pathogen attack (Van Wees *et al.*, 1999; Verhagen *et al.*, 2004). Both WCS417r-ISR and SAR require the NPR1 protein (Pieterse *et al.*, 1998), suggesting that NPR1 is important in regulating and connecting different hormone-dependent defense pathways. Recently, the transcription factor (TF) gene *MYB72* was demonstrated to be required for the onset of WCS417r-ISR (Chapter 2). *MYB72* gene expression is specifically activated in the roots of WCS417r-colonized plants. Analysis of *myb72* knockout mutants revealed that whereas MYB72 is an essential regulator of ISR, it is not involved in SAR.

Many chemicals have been described to trigger similar induced resistance responses. Most of these agents trigger the SAR pathway, as they activate a similar set of *PR* genes, and fail to induce resistance in mutant plants that cannot express SAR (Lawton *et al.*, 1996; Dong *et al.*, 1999). However, the non-protein amino acid ß-amino butyric acid (BABA) has been shown to trigger an, at least partially, different induced resistance response. Like WCS417r-ISR, BABA-induced resistance (BABA-IR) is not associated with a strong transcriptional activation of defense-related genes (Zimmerli *et al.*, 2000; 2001). Instead, BABA primes the plant tissue for enhanced activation of SA-responsive genes, such as *PR-1* (Zimmerli *et al.*, 2000). BABA-IR in *Arabidopsis* against *Pst* DC3000, resembles pathogen-induced SAR in its requirement of SA and NPR1 (Zimmerli *et al.*, 2000). Yet, BABA-IR against *H. parasitica* is fully expressed in *Arabidopsis* genotypes that are impaired in SAR signaling (Zimmerli *et al.*, 2000). This SA- and NPR1-independent form of BABA-IR is based on priming for augmented deposition of callose-rich papillae at the sites of attempted penetration (Zimmerli *et al.*, 2000; Jakab *et al.*, 2001; Ton & Mauch-Mani, 2004).

Screening for *Arabidopsis* mutants that are impaired in BABA-induced sterility (*ibs*) resulted in the isolation of two mutants, *ibs2* and *ibs3*, which are affected in BABA-induced priming for enhanced papillae deposition upon infection by *H. parasitica*. The *ibs2* mutant carries a mutation in the *SAC1b* gene that encodes a polyphosphoinositide phosphatase (Despres *et al.*, 2003a), suggesting involvement of a phosphoinositide-dependent signaling pathway in the regulation of BABA-induced priming for cell wall strengthening (Ton *et al.*, 2005). The *ibs3* mutant, on the other hand, is impaired in the regulation of the zeaxanthin epoxidase gene *ABA1/NPQ2*, which linked ABA signaling to the regulation of BABA-induced priming (Ton *et al.*, 2005). The latter finding was

supported by previous findings that the *aba1-5* mutant that lacks ABA, as well as the ABA response mutant *abi4-1*, are impaired in BABA-induced priming for enhanced papillae deposition after inoculation with the necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina* (Ton & Mauch-Mani, 2004). Hence, BABA-induced priming for enhanced deposition of pathogen-inducible papillae must involve regulation by a phosphoinositide- and ABA-dependent signaling pathway.

Despite the differences in signal-transduction pathways between WCS417r-ISR and BABA-IR, both forms of induced resistance are characterized by primed resistance mechanisms (Van Wees *et al.*, 1999; Zimmerli *et al.*, 2000; Ton & Mauch-Mani, 2004; Verhagen *et al.*, 2004). Although priming has been known for years, the current understanding of the underlying molecular mechanisms remains rudimentary. It has been hypothesized that the induction of priming leads to an increase in the level of signaling components that play a role in basal resistance (Conrath *et al.*, 2006). Because there are hardly any defense mechanisms activated upon induction of priming, it is assumed that these signaling components remain inactive until the plant is exposed to pathogen attack. After perception of a second, pathogen-derived signal, the enhanced signaling capacity in primed plants would facilitate a faster and stronger basal defense reaction. Because primed plants upon pathogen challenge are characterized by a faster and stronger transcriptional induction of defense-related genes (Zimmerli *et al.*, 2000; Kohler *et al.*, 2002; Verhagen *et al.*, 2004; Ton *et al.*, 2007), transcription factors (TFs) may be decisive signaling components, whose expression is enhanced directly upon induction of priming. On the other hand, primed callose deposition is a relatively rapid defense reaction that it is unlikely to be controlled at the transcriptional level, suggesting that transcription-independent mechanisms are involved as well.

To gain insight into the complexity of priming during different forms of induced resistance, we compared two cellular responses that may play an important role in WCS417r- and BABA-induced priming: 1) the formation of callose-rich papillae at the site of pathogen entry, and 2) the activation of TF genes. To study the regulation of the enhanced capacity to form papillae at the site of attempted pathogen entry, we focused on the role of IBS2 and IBS3 in WCS417r-ISR and BABA-IR against *H. parasitica*. Because both WCS417r-ISR and BABA-IR are associated with priming for enhanced defense-related gene expression (Zimmerli *et al.*, 2000; Verhagen *et al.*, 2004; Ton *et al.*, 2005), we studied the involvement of TFs in the onset of the WCS417r- and BABA-induced defense priming. Many plant TFs are thought to be tightly regulated at the transcriptional level (Chen *et al.*, 2002; Lee *et al.*, 2006). However, many TF genes are expressed at such low levels that DNA microarrays are not sufficiently sensitive for a reliable quantification of their expression (Czechowski *et al.*, 2004). Therefore, we used a collection of 2.248 quantitative polymerase chain reaction (Q-PCR) primer sets, described by Czechowski *et al*. (2004), McGrath *et al*. (2005) and Libault *et al*. (2007), to obtain a more sensitive and reliable expression profile of all potential TF genes in the *Arabidopsis* genome upon WCS417r- and BABA-induced priming.

RESULTS

Both WCS417r-ISR and BABA-IR against *H. parasitica* are associated with priming for callose-rich papillae

In *Arabidopsis* WCS417r-ISR is moderately effective in reducing tissue colonization and symptom severity by the oomycete *H. parasitica* (Ton *et al.*, 2002b). However, *H. parasitica* is not sensitive to JA-dependent defenses (Thomma *et al.*, 1998), suggesting that the ISR-mediated protection against this pathogen is not based on WCS417r-induced priming for JA-inducible defense mechanisms. BABA-IR against *H. parasitica* has been shown to involve enhanced formation of callose-containing cell wall appositions at sites of spore germination (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). To examine whether WCS417r-ISR against *H. parasitica* acts through the same priming mechanism, we compared *H. parasitica*-inflicted disease symptoms and the number of spores that induced callose depositions in the epidermal cell layer in WCS417r-ISR- and BABA-IR-expressing plants. As shown in Figure 5.1A, application of BABA protected

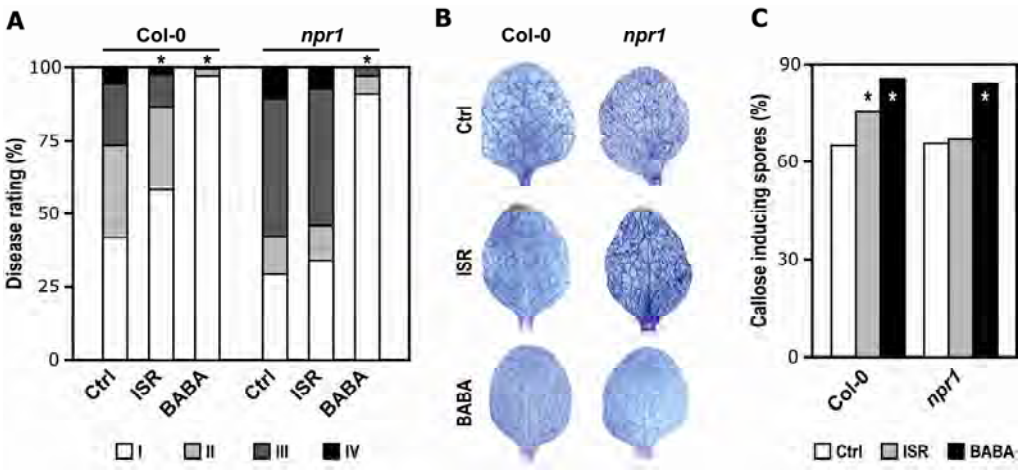

Figure 5.1. Priming for defense during expression of WCS417r-ISR and BABA-IR against *H. parasitica* in *Arabidopsis* Col-0 and *npr1*.

(A) **Quantification of ISR and BABA-IR against *H. parasitica* at eight days after inoculation. ISR was triggered by transferring two-week-old seedlings to potting soil containing *P. fluorescens* WCS417r bacteria. BABA was applied to three-week-old plants by soil-drenching to a final concentration of 80 µM BABA. One week after transplanting into WCS417r-containing soil and one day after soil-drench with BABA, plants were challenged with *H. parasitica* by spraying a suspension of $5x10^4$ spores.mL^{-1} onto the leaves. Disease ratings are expressed as the percentages of leaves in disease classes I (no sporulation), II (trailing necrosis), III (< 50% of the leaf area covered by sporangia), and IV (heavily covered with sporangia, with additional chlorosis and leaf collapse). Asterisks indicate statistically significantly different distributions of disease severity classes compared to the water control (χ^2 test; α=0.05).** (B) **Colonization by the pathogen was visualized by lactophenol/trypan blue staining and light microscopy.** (C) **Quantification of callose deposition at two days after challenge inoculation. Leaves were stained with Calcofluor/aniline blue and analyzed by epifluorescence microscopy (UV). Callose deposition was quantified by determining the percentage of callose-inducing spores in the epidermal cell layer. The data presented are from a representative experiment that was repeated twice with similar results.**

wild-type plants to a greater extent from *H. parasitica* than treatment with WCS417r bacteria. A similar pattern was observed at the level of tissue colonization (Fig. 5.1B). The levels of induced protection by these treatments were correlated with enhanced formation of callose depositions at two days after inoculation with *H. parasitica* (Fig. 5.1C). Hence, both WCS417r and BABA prime for augmented formation of callose-rich papillae.

In contrast to BABA, WCS417r bacteria failed to induce resistance against *H. parasitica* in *npr1* plants. Also here, the level of protection correlated with the priming for papillae formation: whereas WCS417r-treated *npr1* plants failed to show enhanced callose depositions, BABA-treated *npr1* plants showed a similar level of callose priming as wild-type plants (Fig. 5.1). This indicates that WCS417r-priming for cell wall defense against *H. parasitica* depends on NPR1, while the same priming mechanism during BABA-IR functions independently of NPR1. Hence, WCS417r- and BABA-induced priming for enhanced callose deposition are at least partially controlled by different signaling pathways.

Both WCS417r- and BABA-induced priming for enhanced papillae formation require *IBS2* and *IBS3*

BABA-induced priming for enhanced papillae deposition depends on the *IBS2* gene, which encodes a polyphosphoinositide phosphatase, as well as on the *IBS3* gene, which encodes a zeaxanthin epoxidase in the biosynthetic pathway of ABA (Ton *et al.*, 2005). To test whether IBS2 and IBS3 are also involved in WCS417r-induced priming, we inoculated induced and non-induced wild-type, *ibs2* and *ibs3* plants with *H. parasitica* and quantified the numbers of papillae-inducing spores in the epidermal cell layer. At

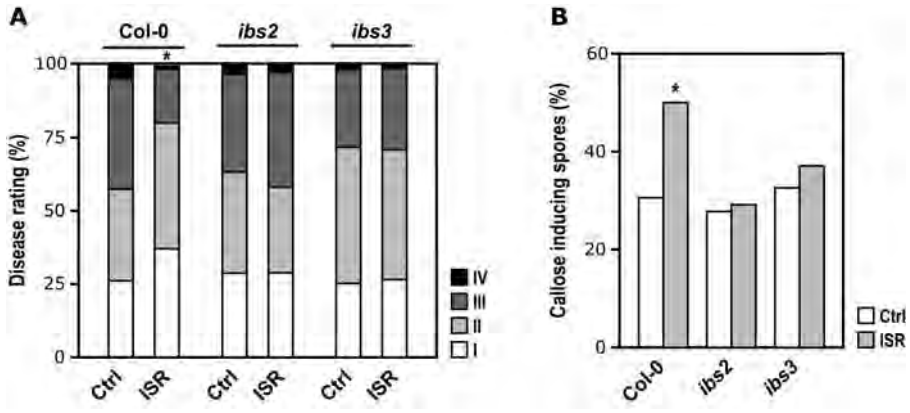

Figure 5.2. Priming for enhanced defense during expression of ISR against *H. parasitica* in *Arabidopsis* Col-0, *ibs2*, and *ibs3*.

(A) **Quantification of WCS417r-ISR against *H. parasitica*.** (B) **Quantification of callose deposition two days after challenge inoculation. For experimental details, see legend of Figure 5.1.**

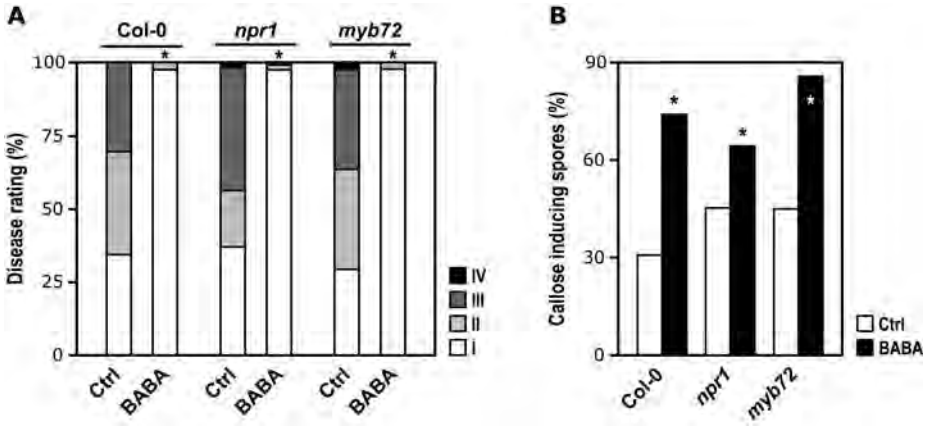

Figure 5.3. Priming for enhanced defense during expression of BABA-IR against *H. parasitica* in *Arabidopsis* Col-0, *npr1*, and *myb72*.

(A) **Quantification of BABA-IR against *H. parasitica*.** (B) **Quantification of callose deposition two days after challenge inoculation. For experimental details, see legend of Figure 5.1.**

two days after challenge inoculation, WCS417r-treated wild-type plants showed an augmented deposition of callose-rich papillae (Fig. 5.2B). In contrast to WCS417r-treated wild-type plants, mutant *ibs2* and *ibs3* plants failed to show this enhanced papillae formation (Fig. 5.2). These data demonstrate that the WCS417r-induced priming for enhanced papillae formation also depends on the *IBS2* and *IBS3* genes. On the other hand, mutants *npr1* and *myb72*, which are both impaired in their ability to express WCS417r-ISR (Pieterse *et al.*, 1998; Chapter 2), were affected neither in BABA-IR (Fig. 5.3A),nor in the BABA-induced priming for enhanced papillae formation (Fig. 5.3B). Hence, WCS417r-ISR and BABA-IR against *H. parasitica* both depend on IBS2 and IBS3, but upstream of IBS2 and IBS3, the WCS417r- and BABA-induced pathways are divergent in their requirement of MYB72 and NPR1.

WCS417r-ISR and BABA-IR against *Pst* DC3000 both function independently of IBS2 and IBS3

To test whether the common requirement of IBS2 and IBS3 for WCS417r-ISR and BABA-IR against *H. parasitica* also applies to the induced resistance against *Pst* DC3000, symptoms in Col-0, *ibs2*, and *ibs3* plants were determined after treatment with WCS417r or BABA. Because both WCS417r-ISR and BABA-IR against *Pst* DC3000 were demonstrated to be NPR1-dependent, mutant *npr1* plants were used as a control. Treatment with WCS417r bacteria resulted in significant disease suppression in Col-0, *ibs2* and *ibs3*, but not in *npr1* (Fig. 5.4A). In agreement with earlier findings (Zimmerli *et al.*, 2000; Ton *et al.*, 2005), a soil drench with 250 µM BABA resulted in a substantial reduction of disease in wild-type, *ibs2* and *ibs3*, but not in *npr1* (Fig. 5.4B). These results demonstrate that neither WCS417r-ISR, nor BABA-IR against *Pst* DC3000 is dependent

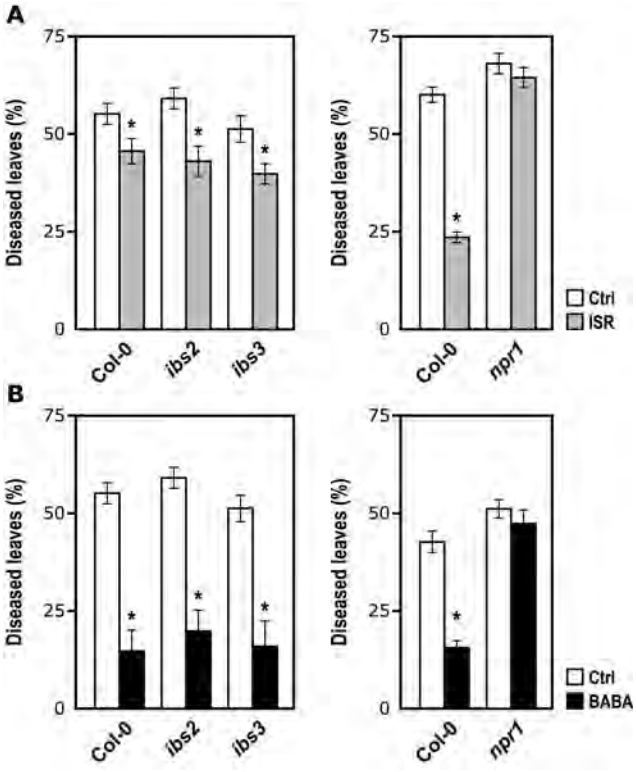

Figure 5.4. WCS417r-ISR and BABA-IR against *Pst* DC3000 in Col-0, *ibs2*, *ibs3* and *npr1*.

(A) **Quantification of WCS417r-ISR. Two-week-old plants were transferred to potting soil containing *P. fluorescens* WCS417r bacteria and three weeks later inoculated with a bacterial suspension of *Pst* DC3000 at $1.25x10^7$ cfu.mL^{-1}. Plants were scored four days after challenge inoculation. Data presented are means of the average percentage of diseased leaves per plant (± SD). Asterisks indicate statistically significant differences compared to non-induced control plants (Student's *t* test; α=0.05; *n*=20-25).** (B) **Quantification of BABA-IR. Five- to six-week-old plants were soil-drenched with BABA to a concentration of 250 µM, and two days later challenge inoculated with *Pst* DC3000. Inoculation and disease scoring were performed as described above.**

on IBS2 and IBS3, while they do require NPR1. These results also indicate that WCS417r- and BABA-induced priming of defense against *Pst* DC3000 operate differently from the priming of papillae formation upon infection with *H. parasitica*.

Both WCS417r and BABA prime for defense-related gene expression

Expression of WCS417r–ISR against *Pst* DC3000 is accompanied by a faster and stronger expression of JA-inducible genes upon pathogen infection, whereas expression of SA-responsive genes is unaltered (Van Wees *et al.*, 1999; Hase *et al.*, 2003; Verhagen *et al.*, 2004). However, endogenous JA levels are not increased as a result of pretreatment with WCS417r (Pieterse *et al.*, 2000). Alternatively, the sensitivity of the tissue to JA may be enhanced. To investigate whether priming by WCS417r is based on increased sensitivity to JA, control and WCS417r-treated plants were tested for the expression of the JA-responsive marker gene *LOX2* (*LIPOXYGENASE 2*) after treatment of the leaves with 100 µM MeJA. As shown in Figure 5.5A, WCS417r-treated plants showed an accelerated induction of *LOX2* in comparison to non-primed control plants. Hence, WCS417r-induced priming is associated with an increase in the responsiveness to JA. This result supports our earlier findings that ISR is predominantly effective against pathogens that are resisted through JA-dependent defense mechanisms (Ton *et al.*, 2002b).

In contrast to WCS417r-ISR, BABA-IR against *Pst* DC3000 is not marked by priming for enhanced expression of JA-responsive genes, but for enhanced expression of SA-responsive genes after pathogen attack (Zimmerli *et al.*, 2000; Van Hulten *et al.*, 2006). To assess whether BABA-induced priming acts through an increase in the sensitivity to SA, water- and BABA-treated plants were sprayed with increasing concentrations of the SA analogue BTH (benzothiadiazole), and subsequently tested for expression of the SA-responsive marker gene *PR-1*. At six hours after application of either 50 or 200 mg.L^{-1} BTH, BABA-treated plants clearly showed an augmented induction of the *PR-1* gene in comparison to non-primed water-treated plants (Fig. 5.5B). In another experiment, a similar increase was found 24 h after treatment with 150 and 300 mg.L^{-1} BTH (Fig. 5.5B). Hence, priming by BABA enhances the sensitivity to SA.

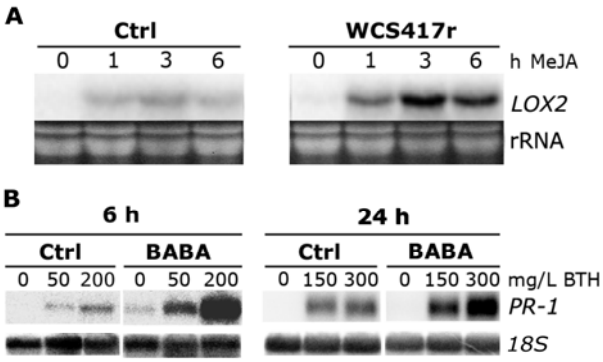

Figure 5.5. Priming for enhanced transcription of defense-related genes in *Arabidopsis* Col-0.

(A) **WCS417r-ISR-related priming for enhanced induction of the MeJA-inducible *LOX2* gene. ISR was triggered by transferring two-weeks-old Col-0 seedlings to soil containing *P. fluorescens* WCS417r bacteria (5x10^{7} cfu.g^{-1}). Shoots of five-weeks-old plants were dipped in a solution containing 100 µM MeJA. Leaf rosettes were harvested at the indicated time points after MeJA treatment.** (B) **BABA-induced priming for enhanced transcription of the *PR-1* gene upon treatment with the SA analogue benzothiadiazole (BTH). Five-weeks-old Col-0 plants were soil-drenched with 250 µM BABA and one day later treated by spraying the indicated concentrations of BTH on the leaves. Leaf material for RNA blot analysis was collected 6 and 24 h later, respectively.**

Systemic changes in TF gene expression upon treatment with WCS417r or BABA

Both WCS417r and BABA induce priming for enhanced transcription of defense-related genes (Fig. 5.5). To investigate whether this enhanced transcriptional activity is based on increased expression of TF genes upon treatment with WCS417r or BABA, the transcription of all potential TF genes in the *Arabidopsis* genome was quantified by Q-PCR, using the collection of 2.248 primer sets described by Czechowski and colleagues (Czechowski *et al.*, 2004; McGrath *et al.*, 2005; Libault *et al.*, 2007). This technique is significantly more sensitive for the detection of small differences in TF gene expression

than our previously used DNA array technology (Czechowski *et al.*, 2004; Verhagen *et al.*, 2004). RNA was extracted from leaves of plants that were grown in soil with or without ISR-inducing WCS417r bacteria and from leaves of water- or BABA-treated plants 32 h after soil-drench treatment. Because BABA primes for enhanced induction of SA/NPR1-dependent genes (Zimmerli *et al.*, 2000) and because priming of pathogen-inducible defense mechanisms in *Arabidopsis* has been shown to require NPR1 (Kohler *et al.*, 2002), we included water- and BABA-treated *npr1* plants in the analysis, in order to distinguish between NPR1-dependent and NPR1-independent priming by BABA. To select for TF genes with altered expression values, we chose an arbitrary cut-off value of two-fold.

In wild-type Col-0 plants, root colonization by WCS417r bacteria caused a more than two-fold induction of 90 TF genes, whereas 31 TF genes were repressed (Fig. 5.6A; Supplementary Table 5.1). Many different types of TFs were induced, with the category of *AP2/ERFs* (*APETALA 2/ETHYLENE RESPONSIVE FACTORs*) notably overrepresented (17 out of 155). Upon soil-drench treatment with BABA, 186 TF genes in leaves of Col-0 plants were more than two-fold induced, whereas 44 TF genes were repressed. Particularly the transcriptional activity of several *WRKY* TF genes was up-regulated (21 out of 71). A similar treatment of *npr1* plants resulted in an enhanced transcription of 135 TF genes, and a repression of 141 TF genes (Fig. 5.6A; Supplementary Table 5.1). Of all BABA-inducible TF genes identified in Col-0 and *npr1* plants, only 32 (10%) were induced in both Col-0 and *npr1* plants (Fig. 5.6B). This indicates a strong influence of NPR1 on the action of BABA on TF gene expression. Furthermore, out of 247 TF genes that were transcriptionally induced by WCS417r or BABA in wild-type plants, only 27 (10.9%)

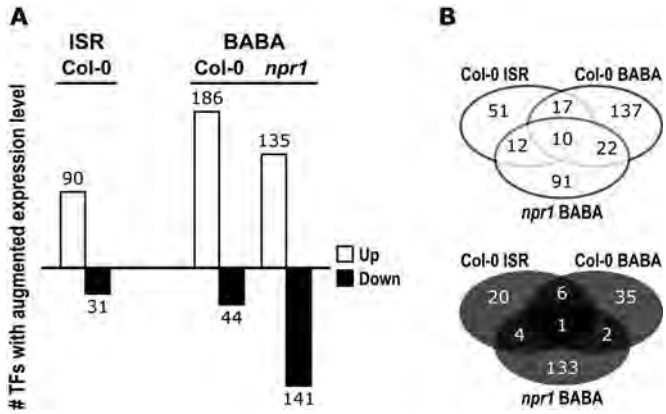

Figure 5.6. Differential TF gene expression in response to WCS417r or BABA in *Arabidopsis* Col-0 and *npr1*. **WCS417r-ISR and BABA-IR were triggered as described in the legend of Figure 5.2. RNA for Q-PCR analysis was extracted from shoot material 11 days after plant transfer to WCS417r-containing soil, or 32 h after soil-drench with BABA. (A) Number of TF genes showing ≥ 2-fold induction (white) or repression (black) in the leaves upon treatment with WCS417r or BABA. (B) Venn-diagrams showing number of overlapping TFs upon treatment with WCS417r or BABA in Col-0 and *npr1*. White diagrams represent induced TF genes, and dark grey diagrams represent repressed TF genes.**

were induced by both WCS417r and BABA. This small overlap corroborates the notion that WCS417r- and BABA prime different sets of defense-related genes (Fig. 5.5).

Expression profiles of selected TFs as markers for priming

To confirm the priming-related induction of TF genes by WCS417r and BABA, we quantified the expression of a dedicated set of 37 TF genes in three biological replicates (Supplementary Table 5.1). The selection was based on the first whole-genome screen, and contained TF genes that showed differential expression under the following conditions: in only wild-type plants after BABA treatment (10 TF genes); in both wild-type and *npr1* plants after BABA treatment (6 TF genes); both in wild-type plants after BABA treatment and in wild-type plants after treatment with WCS417r bacteria (4 TF genes); in both wild-type and *npr1* plants after treatment with BABA, as well as in wild-type plants after treatment with WCS417r bacteria (9 TF genes); or in only wild-type plants after treatment with WCS417r bacteria (8 TF genes). Additionally, we monitored the expression of the TF gene *MYC2*, since this TF gene was shown to play a role in WCS417r-ISR (Chapter 4). As references, seven phytohormone-responsive genes (*PR-1*, *PR-5*, *RAB18*, *PDF1.2*, *LOX2*, *VSP2* and *EBF2*) were included, as well as four constitutively expressed genes (*GAPDH*, *UBI-10*, At1g13320 and At1g62930).

To determine the specificity of the changes in the selected TF-encoding genes, we also included the ISR-non-inducing rhizobacterial strain *Pseudomonas fluorescens* WCS374r and the inactive BABA isomer α-amino butyric acid (AABA) as negative control treatments (Van Wees *et al.*, 1997; Jakab *et al.*, 2001). The resulting expression profiles were subjected to cluster and principal component analyses. As shown in Figure 5.7A, the expression profiles of the 37 TF genes corresponding to the three replicate samples from WCS417r-treated plants formed a distinct cluster separate from control-treated plants and plants treated with the ISR-non-inducing strain WCS374r. This demonstrates that the expression profile of the selected 37 TF genes is sufficiently robust to specifically mark the onset of ISR-dependent priming.

In a separate experiment, the profiles of the three replicate samples from BABA-treated plants formed a distinct cluster compared to the profiles of the treatments with water or inactive AABA (Fig. 5.7B). Additionally, we compared the profiles from the wild type and the *npr1* mutant upon treatment with either water or BABA. The resulting expression profiles clearly differentiated the BABA effects between Col-0 and *npr1* plants (Fig. 5.7C). While confirming our findings from the genome-wide TF-expression profiling (Fig. 5.6), these analyses also demonstrate that WCS417r-ISR and NPR1-dependent and –independent BABA-IR can be clearly distinguished on the basis of the set of 37 TF genes.

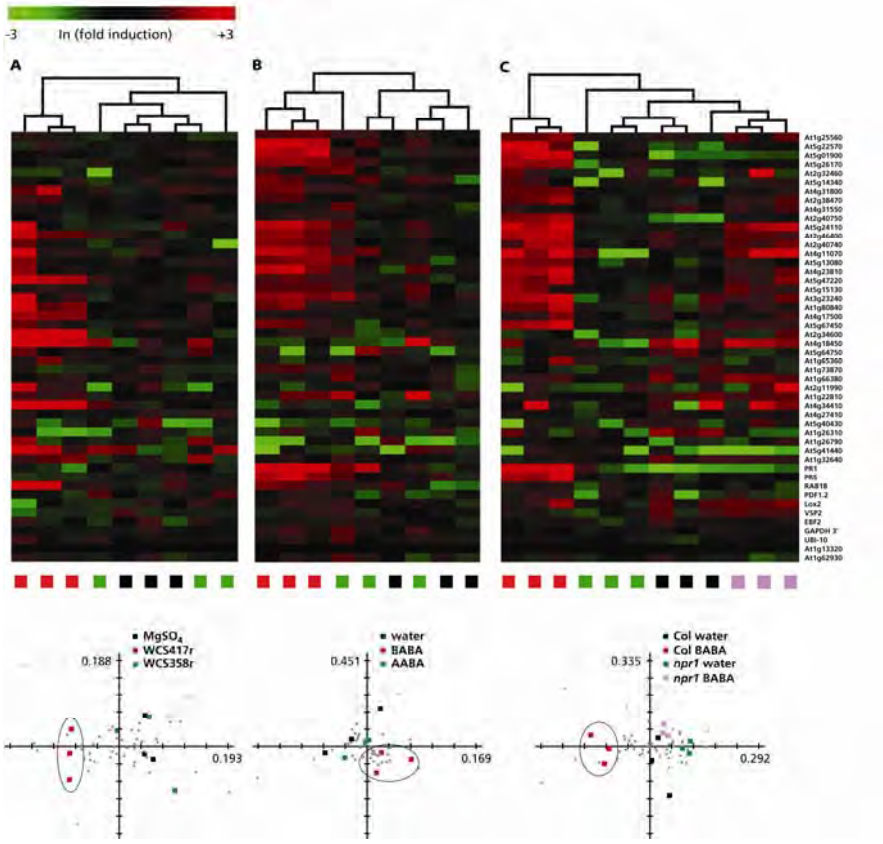

Figure 5.7. **Cluster analysis (top) and principal component analysis (bottom) of the systemic expression of selected TF genes (right) in *Arabidopsis* Col-0 upon treatment with: (A) WCS417r, (B) BABA, or (C) in Col-0 and *npr1* treated with BABA. Root colonization with WCS374r and application of AABA were included as negative controls in (A) and (B), respectively. Color intensity of induced (red) or repressed (green) genes is proportional to the fold induction values of each gene. Fold induction was defined as the expression value in each replicate sample devided by the mean expression value of the three corresponding control samples (water or $MgSO_4$). Ln-transformed fold inductions were subjected to average linkage clustering (Euclidean Distance) and principal component analysis (PCA) using TMEV software (Saeed *et al.*, 2003). Grey dots in the PCA represent the 48 denoted genes, whilst squares represent treatments.**

Promoter analysis of WCS417r- and BABA-inducible TF genes

Transcription of genes is dependent on specific *cis*-acting elements in their promoter regions (Singh *et al.*, 2002). To identify specific elements that may be involved in the priming-related induction of TF genes, we compared 1-kB promoter regions of WCS417r- and BABA-responsive genes using POBO software (Kankainen & Holm, 2004). As shown in Figure 5.8, three distinct elements could be distinguished that were statistically over-represented in the promoters of WCS417r-inducible TF genes in Col-0, BABA-inducible TF genes in Col-0, or BABA-inducible TF genes in *npr1* plants. All three

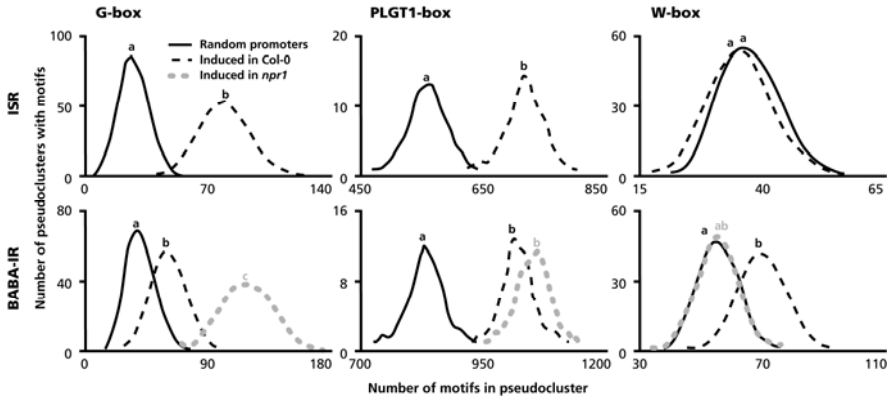

Figure 5.8. Occurrence of *cis*-acting elements in the promoter regions of WCS417r- and BABA-inducible TF genes in *Arabidopsis* Col-0 and *npr1*. **Occurrences of G-box (CACGTG), PLGT1-box (GAAAAA), and W-box (TTGACC) motifs were quantified in the 1000-bp sequences preceding the 5'-end of each transcription unit, using POBO bootstrapping analysis (Kankainen & Holm, 2004). The WCS417r- and BABA-inducible TF genes in Col-0 (black dash) and *npr1* (grey dash) were compared to randomly selected promoter sequences (black undashed) from the *Arabidopsis* genome. Different letters indicate statistically signicant differences in the occurrence of the of the *cis*-acting element (χ^2 test; α=0.05).**

groups of promoter sequences were enriched in G-box and PLGT1-box elements, which are associated with responses to pathogen infection and salt stress (Dröge-Laser *et al.*, 1997; Faktor *et al.*, 1997; Boter *et al.*, 2004; Park *et al.*, 2004). Notably, the promoter regions of the BABA-responsive TF genes in *npr1* displayed a stronger enrichment in G-box elements than those in wild-type plants (Fig. 5.8). This indicates that the G-box element is mostly involved in the NPR1-independent induction of TF genes by BABA. Furthermore, the promoter regions of BABA-responsive TF genes in Col-0 plants showed a statistically significant enrichment in WRKY-binding W-box elements, whereas this over-representation was absent in the WCS417r-responsive promoters of Col-0, and the BABA-responsive prometers of *npr1* plants (Fig. 5.8). These observations suggest that WRKYs are involved in the NPR1-dependent induction of TF genes by BABA.

Identification of a novel promoter element in BABA-inducible *WRKY* TF genes

The group of 187 BABA-inducible TFs contains 21 *WRKY* genes. Many of these, such as *WRKY18*, *WRKY38*, *WRKY58*, *WRKY59*, and *WRKY70*, have been associated with SA-dependent defenses and were recently identified as direct targets of NPR1 (Wang *et al.*, 2006). Indeed, the induction of 20 out of the 21 BABA-responsive *WRKY* genes was blocked or strongly reduced in the *npr1* mutant (Fig. 5.9). To further investigate the regulation of this NPR1-dependent induction of *WRKY* genes, we examined the BABA-inducible *WRKY* genes for overrepresentation of so-far unknown promoter elements. Using the Statistical Motif Analysis tool of TAIR http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp), we found a very significant over-representation of two nearly

identical motifs, TAGTCT and TAGACT (binomial distribution; $P = 3.81e^{-05}$ and $P = 1.63e^{-04}$, respectively). Subsequently, we performed POBO analysis to compare the frequency distributions of these DNA motifs between promoters of BABA-inducible *WRKY* genes, promoters of BABA non-inducible *WRKY* genes, and a set of random *Arabidopsis* promoters. Although both elements were significantly over-represented in the BABA-inducible *WRKY* promoters (data not shown), the most contrasting differences between the three different sets of promoters was found for the combined TAG[TA]CT motif (Fig. 5.9). The fact that this motif is strongly over-represented in the BABA-inducible *WRKY* promoters ($\chi^2 = 14.47$; $P < 0.001$), but not in the BABA non-inducible *WRKY* promoters ($\chi^2 = 0.064$; $P = 0.8$), points to the occurrence of a specific regulatory factor in the BABA-induced activation of *WRKY* genes.

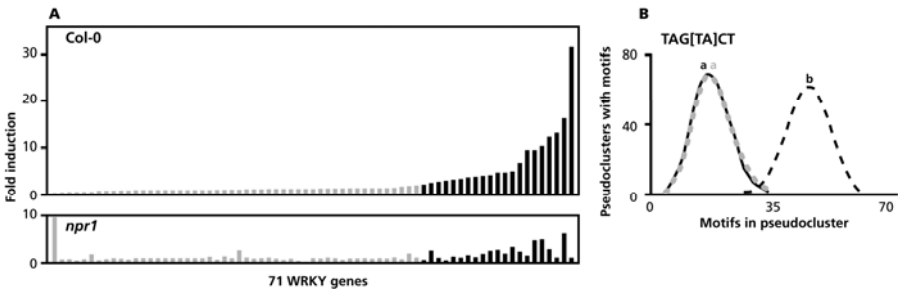

Figure 5.9. Identification of a specific element in the promoter regions of BABA-inducible, NPR1-dependent *WRKY* genes. (A) **Fold inductions of 71 *Arabidopsis WRKY* TF-genes in response to BABA in Col-0 and *npr1*.** (B) **Occurrences of the** TAG[TA]CT **motif in promoter regions of BABA-response *WRKY* genes (black dashed), BABA-nonresponsive WRKY genes (grey dashed), and random *Arabidopsis* promoters (black undashed). Different letters indicate statistically signicant differences in occurrence of the** TAG[TA]CT **motif (χ^2 test; α=0.05).**

DISCUSSION

Activation of WCS417r-ISR and BABA-IR is not associated with a direct induction of defense mechanisms, but with priming for augmented defense induction after pathogen attack (Conrath *et al.*, 2006). Since the two forms of induced resistance are effective against a partly overlapping spectrum of pathogens, it is plausible to assume that WCS417r bacteria and BABA prime for distinct, yet overlapping, defense reactions. In this study, we investigated differences and similarities in WCS417r- and BABA-induced priming for enhanced callose deposition and defense-related gene expression. A clear difference between WCS417r- and BABA-induced priming is that they target distinct classes of defense-related genes: whereas WCS417r primes for enhanced transcription of

JA-inducible genes, such as *LOX2*, BABA primes for enhanced transcription of SA-inducible genes, such as *PR-1* (Fig. 5.5; Chapter 4; Verhagen *et al.*, 2004; Ton *et al.*, 2005). On the other hand, WCS417r and BABA both prime for enhanced formation of callose-rich papillae during infection by the oomycete *H. parasitica* (Figs. 5.1 – 5.3). This WCS417r- and BABA-induced priming for cell wall strengthening was impaired in mutants *ibs2* and *ibs3* (Fig. 5.2), indicating that WCS417r-ISR and BABA-IR against this pathogen both involve ABA- and –phosphoinositide-dependent defenses. Mutants *ibs2* and *ibs3* were not impaired in the expression of WCS417r-mediated ISR against the bacterial pathogen *Pst* DC3000, nor was BABA-IR (Fig. 5.4). Clearly, defense against this bacterium requires (a) different mechanism(s). Bacteria enter leaves through natural openings and are thus not restricted by cell wall barriers.

Based on our observations that WCS417r and BABA prime for enhanced transcription of JA- and SA-inducible genes, respectively (Fig. 5.5), we decided to examine wether activation of WCS417r- and BABA-induced priming is associated with enhanced expression of TF genes. To this end, we screened the transcriptional response of all potential TF genes in the *Arabidopsis* genome in response to activation of WCS417r-mediated ISR and BABA-IR. Previously, Verhagen *et al.* (2004) were unable to detect transcriptional alterations in the leaves of *Arabidopsis* plants with ISR elicited by root treatment with WCS417r, whereas we found consistent effects on TF gene expression (Figs. 5.6 & 5.7). This apparent discrepancy can be explained by the use of different methods. Whereas Verhagen *et al.* (2004) used micro-arrays to quantify gene expression, the transcriptional profiling in this study was based on Q-PCR. This latter technique is substantially more sensitive and reliable for detection of low-abundant mRNAs, characteristic for the expression of TF genes (Czechowski *et al.*, 2004).

Instead, Q-PCR revealed that treatment of the roots with either WCS417r or BABA induced major changes in TF gene expression in the leaves (Fig. 5.6A) with little overlap (Fig. 5.6B). Hence, priming by WCS417r or BABA is associated with transcriptional responses of largely distinct sets of TF genes. In combination with our previous findings that WCS417r and BABA prime for different sets of defense-related genes (Fig. 5.5; Chapter 4; Verhagen *et al.*, 2004; Ton *et al.*, 2005), we can propose that WCS417r-induced changes in TF gene expression contribute to priming of JA-inducible genes, whereas the BABA-targeted TF genes contribute to priming of SA-inducible genes (Fig. 5.10). In agreement with this idea, WCS417r bacteria were found to induce the expression of TF genes related to the regulation of JA- and ET-dependent defense reactions (Supplementary Table 5.1), including 17 *AP2/ERF* genes. Amongst these, the *ERF1* (At3g23240) encodes a key regulator in the integration of JA- and ET-dependent signaling pathways (Lorenzo *et al.*, 2003). ERF1-dependent activation of defense-related genes has been reported to be counteracted by an ABA/JA-inducible signaling pathway, which requires MYC2 (Lorenzo *et al.*, 2004). The *MYC2* gene (At1g32640) was also weakly, yet consistently, induced in the leaves after treatment with WCS417r (Fig. 5.7A; Supplementary Table 5.1). Also in line with our hypothesis, BABA induced a relatively

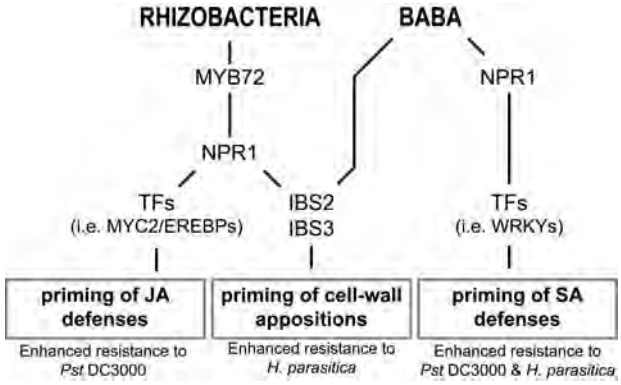

Figure 5.10. WCS417r- and BABA-induced priming for defense against *Pst* DC3000 and *H. parasitica*. **Root colonization by WCS417r bacteria renders *Arabidopsis* plants more resistant against *Pst* DC3000 and *H. parasitica* through a MYB72 and NPR1 dependent mechanism. ISR against *Pst* DC3000 is based on a primed responsiveness of JA-dependent defenses. Treatment with ISR-inducing bacteria leads to the induction of *MYC2* and *ERF/EREBP* TFs. WCS417r also triggers IBS2- and IBS3-dependent priming for enhanced callose deposition at *H. parasitica* entry sites. Both these responses are dependent on NPR1. Treatment with BABA also primes *Arabidopsis* defense mechanisms that are effective against *Pst* DC3000 and *H. parasitica*. However, BABA-IR against *Pst* DC3000 depends on SA/NPR1 signaling. In contrast to WCS417r-ISR, downstream of NPR1 *WRKY* TFs are activated, which fine-tune the expression levels of downstream SA-dependent effector genes. Furthermore, treatment with BABA generates IBS2- and IBS3-dependent, but NPR1-independent priming for callose deposition and resistance against *H. parasitica*.**

large set of TF genes, of which the majority was no longer inducible in the *npr1* mutant (Figs. 5.6 & 5.7). Hence, NPR1 is required for the BABA-induced expression of many TF genes, including 20 members of the *WRKY* family. Many of these genes, such as *WRKY18*, *WRKY38*, *WRKY58*, *WRKY59*, and *WRKY70*, have been reported to play an important role in the fine-tuning of SA-inducible defenses (Eulgem, 2005), and were recently identified as direct targets of NPR1 (Wang *et al.*, 2006).

The small number of overlapping TFs might act as regulators of defense mechanisms that are primed by both WCS417r and BABA treatment, such as formation of callose-containing cell-wall appositions. Enhancement of this defense response is dependent on IBS2 and IBS3, regardless of the nature of the resistance inducing treatment. Yet, this response is regulated differently at the level of NPR1. NPR1 is required for WCS417r-mediated priming of papillae-formation, but the protein is dispensable for priming of defense responses upon treatment with BABA. Thus, it must be concluded that NPR1 acts upstream of IBS2 and IBS3 in WCS417r-induced priming for enhanced callose deposition, whereas it does not play a role in the same response induced by BABA (Fig. 5.10).

The direct effects of WCS417r and BABA on TF gene expression point to specific signaling pathways that regulate the onset of priming through enhanced expression of defense-related TFs. These priming-related TF genes must be controlled by other TFs that may not be regulated at the transcriptional level. Such "early-acting" TFs in the priming

pathway may act as key regulators in the onset of priming for enhanced defense-related gene induction. In a first step to identify such factors, we analyzed the promoter regions of WCS417r-inducible and BABA-inducible TFs for their common *cis*-acting elements. The promoter regions of both WCS417r- and BABA-inducible TF genes were significantly enriched in G- and PLGT1-boxes (Fig. 5.8). Both these elements have been related to transcriptional responses to pathogen infection, salt stress, JA, and ABA (Dröge-Laser *et al.*, 1997; Faktor *et al.*, 1997; Boter *et al.*, 2004; Park *et al.*, 2004). The promoter regions of BABA-inducible genes in the *npr1* mutant displayed a much stronger enrichment in G-box elements than those in wild-type plants (Fig. 5.7). Apparently, disruption of the NPR1-dependent signaling pathway results in an enhanced activation of G-box-containing TF genes by BABA. This suggests that in wild-type plants, NPR1 suppresses BABA-induced expression of G-box-containing TF genes.

In the wild-type, but not in the *npr1* mutant, we found a statistically significant overrepresentation of W-boxes in the promoter regions of BABA-inducible genes. This points to an involvement of WRKY proteins in the NPR1-dependent induction of TF genes by BABA, and is supported by our finding that 21 *WRKY* genes were more than two-fold induced by BABA. Further analysis of the promoter regions of these 21 *WRKY* genes revealed a significant overrepresentation of an, as yet uncharacterized, promoter element (Fig. 5.9). We hypothesize that this TAG[TA]CT element functions as an important *cis*-acting element in the NPR1-dependent activation of TF genes by BABA. Future studies will focus on the identification of TF proteins that bind to the TAG[TA]CT element, aiming at the identification of novel key regulators in the priming for SA-dependent defense mechanisms.

EXPERIMENTAL PROCEDURES

Cultivation of plants

Seeds of *Arabidopsis thaliana* accession Col-0 and its mutants *ibs2-2,* which carries a T-DNA insertion in the 5′-untranslated region of *SAC1b* (At5g66020; this mutant is also referred to as s-031243; Ton *et al.*, 2005), *ibs3-2*, which harbors an EMS-induced mutation in the *ABA1*/*NPQ2* gene; (also referred to as *npq2-1*; Niyogi *et al.*, 1998; Ton *et al.*, 2005), *myb72-1* (Chapter 2) and *npr1-1* (Cao *et al.*, 1994) were sown in quartz sand. Ten days after germination, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min with a 24-h interval. Plants were cultivated in a growth chamber with an eight-h day (200 $\mu Em^{-2}.sec^{-1}$ at 24°C) and 16-h night (20°C) cycle at 70% relative humidity for another 11 days. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland & Arnon, 1938) containing 10 μM Sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

Cultivation of micro-organisms

For treatment of the roots with ISR-triggering rhizobacteria, the rifampicin-resistant *Pseudomonas fluorescens* strain WCS417r (Pieterse *et al.*, 1996) was grown on King's medium B agar plates (King *et al.*, 1954) for 24 h at 28°C. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$ to a density of 10^9 colony-forming units (CFU) per mL.

The virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Whalen *et al.*, 1991), was cultured overnight in liquid King's medium B at 28°C, collected by centrifugation, and resuspended in 10 mM $MgSO_4$ to a final density of 2.5×10^7 CFU.mL^{-1}.

Hyaloperonospora parasitica WACO9 was obtained from the Plant Research Institute, Wageningen, The Netherlands. The oomycete was maintained on susceptible WS-0 plants as described by Koch & Slusarenko (1990). Sporangia were obtained by washing heavily diseased leaves in 10 mM $MgSO_4$, collected by centrifugation, and resuspended in 10 mM $MgSO_4$ to a final density of 5×10^4 spores.mL^{-1}.

Induction of systemic resistance

ISR was elicited by transplanting two-week-old seedlings into a sand/potting soil mixture containing 5×10^7 CFU WCS417r bacteria per gram of soil. Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. BABA-IR was triggered by applying BABA (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) as a soil drench at the indicated concentrations.

Chemical treatments

Treatment with methyl jasmonate (MeJA) was performed by dipping five-week-old Col-0 plants in an aqueous solution containing 100 µM MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands) and 0.015% Silwet L-77 (Van Meeuwen Chemicals B.V., Weesp, the Netherlands), as described previously (Pieterse *et al.*, 1998). BTH (benzothiadiazole; CIBA-GEIGY GmbH; Frankfurt, Germany) was administered by spraying leaves of five-week-old plants with a BTH solution containing 0.015% Silwet L-77 (Van Meeuwen Chemicals B.V., Weesp, the Netherlands). Leaf rosettes were harvested at indicated intervals after application and immediately frozen in liquid nitrogen.

P. syringae pv. *tomato* DC3000 bioassays

One day before inoculation with *Pst* DC3000, five-week-old plants were placed in 100% relative humidity. Plants were inoculated by dipping the leaves in a suspension of virulent *Pst* DC3000 bacteria in 10 mM $MgSO_4$ and 0.015% Silwet L-77 (Van Meeuwen

Chemicals, Weesp, The Netherlands). Four days after challenge inoculation, the percentage of leaves with symptoms per plant was determined. Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased. Experiments were conducted with 20-25 plants per treatment and repeated at least once.

H. parasitica bioassays

Three-week-old plants were misted with a *H. parasitica* WACO9 spore suspension in water. Inoculated plants were maintained at 17°C and 100% relative humidity for 24 h. Subsequently, humidity was lowered to 70% to avoid direct effects on plant development and to reduce the chance of secondary infections by opportunistic pathogens. Seven days after challenge inoculation humidity was raised again to 100% to induce sporulation. Disease symptoms were scored for about 250 leaves per treatment at nine days after inoculation. Disease ratings were expressed as intensity of disease symptoms and pathogen sporulation on each leaf: I, no symptoms; II, trailing necrosis; III, < 50% of the leaf area covered by sporangia; IV, heavily covered with sporangia, with additional chlorosis and tissue collapse. To visualize trailing necrosis, infected leaves were stained with lactophenol-trypanblue and examined microscopically at five days after inoculation as described by Koch & Slusarenko (1990).

Quantification of callose-containing papillae

Quantification of callose deposition was performed as described by Ton & Mauch-Mani (2004). In short, leaves were collected at two days after inoculation and incubated overnight in 96% ethanol. Destained leaves were washed in 0.07 M phosphate buffer, pH 9, incubated for 15 min in 0.07 M phosphate buffer containing 0.005% Calcofluor (fluorescent brightener; Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and 0.01% aniline blue (water blue; Merck, Darmstadt, Germany), and subsequently washed in 0.07 M phosphate buffer containing only 0.01% aniline blue to remove excess Calcofluor. Observations were performed with a fluorescence microscope with UV filter (bandpass 340 to 380 nm, long-path 425 nm). Callose depositions were quantified by determining the percentage of callose-inducing spores per infected leaf.

Northern blot analysis

Total RNA was extracted from pooled shoot samples as described by Van Wees *et al.* (1999). For northern blot analysis, 10 µg RNA was denatured using glyoxal and DMSO (Sambrook *et al.*, 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-N^+ membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. Northern blots were hybridized with gene-

specific probes for *PR-1* and *LOX2* as described previously (Pieterse *et al.*, 1998). To check for equal loading, RNA gel blots were stripped and hybridized with a gene-specific probe for 18S rRNA.

Transcription profiling

Q-PCR analysis was performed basically as described by Czechowski *et al.* (2004). For the initial profiling of all putative *Arabidopsis* transcription factors, 200 µg of RNA per treatment from a single experiment was digested with Turbo DNA-free™ (Ambion, Huntingdon, United Kingdom) according to the manufacturer's instructions. To check for contamination with genomic DNA, a PCR with primers designed for *EIL2* (At5g21120; $EIL2_F$ and $EIL2_R$; Supplementary Table 5.2) was carried out. DNA-free total RNA was converted into cDNA using oligo-dT_{20} primers (Invitrogen, Breda, the Netherlands), 10 mM dNTPs, and SuperScript™ III Reverse Transcriptase (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Efficiency of cDNA synthesis was assessed by Q-PCR, using primers of the constitutively expressed gene *UBI10* (At4g05320; $UBI10_F$ and $UBI10_R$; Supplementary Table 5.2) and of both the 5′ and 3′ termini of GAPDH; (At1g13440; $GAPDH5'_F$ and $GAPDH5'_R$; $GAPDH3'_F$ and $GAPDH3'_R$; Supplementary Table 5.2). Based on the results, cDNA of each sample was diluted to obtain a *UBI10* C_T (threshold cycle) value of 18 ± 0.5.

PCR reactions, including all TFs, were performed with an ABI PRISM® 7900 HT sequence detection system, using SYBR® Green to monitor the synthesis of double-stranded DNA. One µl of cDNA was mixed with 5 µl 2x SYBR® Green Master Mix reagent (Applied Biosystems), after which 200 nM of a TF-specific primer pair was added (Czechowski *et al.*, 2004; 2005) to a total volume of 10 µl. The following standard thermal profile was used for all PCR reactions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon dissociation curves, i.e. melting curves, were recorded after cycle 40 by heating from 60°C to 95°C with a ramp speed of 1.9°C min^{-1}.

Data were analyzed using the SDS 2.2.1 software (Applied Biosystems). To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) as a function of cycle number, baseline data were collected between cycles 3 and 15. All amplification plots were analyzed with an R_n threshold of 0.1 to obtain C_T values. PCR efficiency (E) was estimated from the data obtained from the exponential phase of each individual amplification plot and the equation $(1 + E) = 10^{slope}$ (Ramakers *et al.*, 2003). To determine normalized TF expression levels (ΔC_T), the C_T of the constitutively transcribed genes At4G05320 (*UBI10*), At2G28390 (*SAND* family), At5G46630 (*CLATHRIN ADAPTOR COMPLEX SUBUNIT*) and At5G55840 (encoding a pentatricopeptide repeat-containing protein) (Czechowski *et al.*, 2005) were individually subtracted from that of the TF of interest, resulting in four different values. Expression ratios are presented as $(1 + E)^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (\Delta C_{T\ Treat}) - (\Delta C_{T\ Ctrl})$. TFs were only considered as induced

or repressed when $\Delta\Delta C_T$, normalized to all four constitutive genes, was ≥ 2 or ≤ 0.5, respectively.

To confirm the results of the transcription profiling, 37 TF genes were selected representing TFs responsive to both, or either of the two induction treatments (Supplementary Table 5.1). Expression levels were checked in three replicate biological samples with an MyIQ™ Single Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands) in combination with SYBR® Green. Five µg of RNA from independent experiments was used for DNase treatment and subsequent cDNA synthesis as described above. PCR reactions were done in optical 96-well plates in a total volume of 15 µl, containing cDNA, 0.5 µL of each of the two gene-specific primers (10 pmol.µL^{-1}), and 3.5 µL of 2x IQ SYBR® Green Supermix reagent. The following PCR program was used for all PCR reactions: 95°C for 3 min; 40 cycles of 95°C for 30 sec, 59.5°C for 30 sec, and 72°C for 30 sec. C_T values were calculated using Optical System Software, version 1.0 for MyIQ™ (Bio-Rad, Veenendaal, the Netherlands). Subsequently, C_T values were normalized for differences in dsDNA synthesis using those of the constitutively expressed reference gene At1g13320 (Czechowski *et al.*, 2005). Melting curves were recorded after cycle 40 by heating from 55°C to 95°C with a ramp speed of 1.9°C min^{-1}. Expression values relative to the reference gene were calculated from $(1 + E)^{\Delta CT}$, where $\Delta C_T = C_T$ (TF gene) - C_T (At1g13320).

Statistical analysis of expression data

Cluster analysis (Euclidean distance) and principal component analysis (PCA) of the transcriptional patterns of the selected TF genes were based on the expression values from three independent biological samples per treatment, using TIGR Multiexperiment Viewer (TMEV) software (Saeed *et al.*, 2003). Both analyses were performed with the Ln-transformed values of the fold induction ratio of each gene, which was defined as the expression value in each replicate sample devided by the mean expression value of the three corresponding control samples.

Promoter analysis

Promoter analyses were based on the 1000 bp sequences preceding the 5'-end of each transcription unit, which were obtained from the Sequence Bulk Download and Analysis tool of TAIR (http://www.arabidopsis.org/tools/bulk/sequences/index.jsp), The promoter sequences of WCS417r- or BABA-inducible TFs were examined for over-representation of *cis*-acting elements, using the POBO bootstrapping program (Kankainen & Holm, 2004). Sequences of 31.351 randomly selected *Arabidopsis* promoters were used as a reference frame. Each analysis was performed under the program settings as recommended at (http://ekhidna.biocenter.helsinki.fi/poxo/pobo/help#p2).

Differences in DNA element frequency between selected groups of promoters were statistically analyzed with a Mantel-Haenszel Chi-square test by comparing proportions

between *1)* the total number of promoters in the selected group *2)* the number of promoters containing the DNA element, and *3)* the total number of DNA elements, using SPSS 11.5 software. The TAG[TA]CT motif in the promoter regions of the BABA-responsive WRKY genes was identified by means of the Statistical Motif Analysis tool of TAIR (http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp).

SUPPLEMENTARY MATERIAL

Supplementary Table 5.1. MS Excel file containing all TF genes > 2-fold induced (TAB 1) or repressed (TAB 2) by the different treatments treatment in leaves of *Arabidopsis* Col-0 or *npr1*, and fold inductions of the dedicated set of TF genes in three biological replicate samples of the different treatments (TAB 3).

Supplementary Table 5.2. Primers used for confirmation of the degradation of genomic DNA after DNase treatment and for normalization of cDNA quantities.

All supplementary materials can be downloaded from:
http://www.bio.uu.nl/~fytopath/GeneChip_data.htm

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CHAPTER 6

General Discussion

Plants are well equipped to respond to changes in their environment. By using complex signaling networks, they have the ability to adapt to changing environmental conditions such as temperature, drought and light availability. Similarly, plants have sophisticated mechanisms to tightly regulate defense responses in order to fend off attacking pathogens and insects in a cost-efficient manner. Once a plant has encountered a pathogen, recognition can lead to systemic enhancement of the host's defensive capacity against future attack, a phenomenon known as systemic acquired resistance (SAR) (Durrant & Dong, 2004). Colonization of roots by non-pathogenic rhizobacteria also induces systemic resistance against various attackers in diverse plant species (Van Loon & Bakker, 2006). This so-called rhizobacteria-induced systemic resistance (ISR) partially differs from SAR in its spectrum of effectiveness and in its signal transduction pathway (Pieterse *et al.*, 1998; Ton *et al.*, 2002b). Apart from the requirements of JA and ET signaling and NPR1, no other components of the signal-transduction pathway underlying rhizobacteria-mediated ISR have been identified. Expression profiling of *Arabidopsis* roots indicated that colonization by WCS417r directly changed the transcriptional activity of 97 genes out of the ~8000 tested (Verhagen *et al.*, 2004). Based on this observation, the first part of the research described in this thesis focused on the involvement of one of the locally WCS417r-induced genes, encoding the transcription factor (TF) MYB72.

ONSET OF ISR

In contrast to disruptions in the gene encoding THAUMATIN LIKE PROTEIN 1 (*TLP1*), of which the transcription was also induced in *Arabidopsis* roots colonized by WCS417r, T-DNA insertions in the *MYB72* gene resulted in a loss of the ability to generate WCS417r-ISR against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), *Hyaloperonospora parasitica*, *Alternaria brassicicola* and *Botrytis cinerea* (Chapter 2; Léon-Kloosterziel *et al.*, 2005). The involvement of MYB72 is restricted to ISR, because other forms of induced resistance, such as SAR (Chapter 2) and BABA-IR (Chapter 5) were unaffected in *myb72* knockout plants.

Previously, Knoester *et al.* (1999) performed bioassays in which ET response mutants of *Arabidopsis* were tested for their ability to express WCS417r-ISR. These experiments revealed that ET is required not only systemically, but also at the site of bacterization (Knoester *et al.*, 1999). To test whether ET regulates *MYB72* expression, its transcription was checked upon application of the ET precursor ACC (Chapter 2). In contrast to the ET-responsive *EBF2* gene, *MYB72* mRNA levels did not increase in response to ACC treatment. Moreover, WCS417r-induced *MYB72* expression was not impaired in the ET-insensitive *ein2* mutant plants. Hence, WCS417r-induced expression of *MYB72* expression is not dependent on ET, which suggests that this response to WCS417r bacteria takes place before the involvement of, or in parallel with, the ET-dependent step in the ISR pathway. Because *MYB72* transcription is induced locally, the corresponding

TF protein must act relatively early in the ISR-signaling pathway. This also suggests that WCS417r-induced *MYB72* expression in the roots is important for the generation of the long-distance signal that triggers the primed state of ISR in the upper leaves. Future research on the downstream target genes of MYB72 should shed more light on the regulatory role of this transcription factor in ISR.

MYB72 induction by itself is not sufficient to trigger ISR, because transgenic plants with constitutively elevated levels of *MYB72* failed to display higher levels of pathogen resistance (Chapter 2). This indicates that the activation of at least one more component is required for the generation of the ISR signal. In a large-scale yeast-two-hybrid screen designed to study possible interactions between *Arabidopsis* TFs, a putative candidate was identified. MYB72 interacted *in vitro* with EIL3, a protein showing homology to EIN3, which is a crucial component in ET-signaling (Chapter 2). EIN3 and its paralogs, the EIN3-like (EIL) proteins, regulate downstream ET-signaling events through the binding to promoter regions of ET-responsive genes, such as ERF1 (ET RESPONSE FACTOR 1) (Chao *et al.*, 1997; Solano *et al.*, 1998). It is tempting to speculate that the local requirement for ET in the roots can be traced to EIL3. Therefore, future challenges are to determine whether the interaction between MYB72 and EIL3 also occurs *in planta*, whether this interaction is dependent on ET signaling, and, most importantly, whether this interaction is required and sufficient to trigger ISR.

TRICHODERMA-MEDIATED ISR

Besides non-pathogenic rhizobacteria, many other soil-borne micro-organisms are known to positively stimulate plant growth. In particular mycorrhizal fungi and rhizobia have been shown to stimulate plant growth through increasing the availability of growth-limiting nutrients (Waters *et al.*, 1998; Spaink, 2000; Harrison, 2005). Other micro-organisms have been shown to stimulate growth indirectly, by antagonizing potentially pathogenic soil pathogens through competition for nutrients, secretion of antibiotic compounds and lytic enzymes, or degradation of components that are important for the pathogen's invasive activity. For *Trichoderma* spp. all these characteristics have been described (Harman *et al.*, 1981; Chet, 1987; Schirmböck *et al.*, 1994; Lorito *et al.*, 1996; Zimand *et al.*, 1996; Woo *et al.*, 1999). Furthermore, recent studies have revealed that root colonization by different *Trichoderma* isolates can enhance the defensive capacity of cucumber plants against various pathogens (Yedidia *et al.*, 2003; Shoresh *et al.*, 2005; Segarra *et al.*, 2007). Moreover, application of the chemical inhibitors silver thiosulfate and diethyldithiocarbamate, which block the action of ET and the synthesis of JA, respectively, reduced these protective effects (Shoresh *et al.*, 2005), This suggesting that the signal transduction pathway of *Trichoderma asperellum* T203-mediated ISR resembles the pathway of *P. fluorescens* WCS417r-mediated ISR in *Arabidopsis*. However, application of high densities of *T. asperellum* T34 inoculum has been reported to trigger SAR-like responses, as evidenced by accumulation of SA and direct activation of defense responses in distal plant parts (Segarra *et al.*, 2007).

To elucidate the signal transduction pathway underlying *Trichoderma*-induced resistance, the ability of T34 to enhance resistance against *Pst* DC3000 was assessed in *sid2* and *myb72* mutants of *Arabidopsis*, which are disturbed in SAR and in ISR signaling, respectively (Nawrath & Métraux, 1999; Ton *et al.*, 2002a; Chapter 2). These experiments showed that root colonization by T34 resulted in systemic protection against *Pst* DC3000. This reduction in disease symptoms by *Pst* DC3000 could also be triggered in *sid2*, but not in *myb72* plants, indicating that T34-mediated ISR is similarly regulated as WCS417r-ISR (Chapter 3). This conclusion was further supported by the finding that root colonization by T34 does not result in direct activation or priming of SA-inducible *PR-1* gene expression, but rather causes a systemic priming for enhanced MeJA-inducible expression of *LOX2* (Chapter 3).

SENSING RHIZOBACTERIA-MEDIATED NUTRIENT DEPLETION

Although various bacterial components have been shown to trigger ISR (Bakker *et al.*, 2007; Newman *et al.*, 2007), it is not known how these determinants are perceived and give rise to ISR. An alternative to direct recognition of ISR-inducers by the plant is the perception of micro-organism-induced alterations in the plant's immediate environment, i.e. the rhizosphere. As both the host plant and the root-colonizing bacteria utilize nutrients for their primary metabolism, some of those might become limiting and give rise to the activation of adaptive responses in the plant. Studies by O'Hara *et al.* (1987) and Mirleau *et al.* (2005) have already indicated that this occurs for readily available inorganic sulfur-containing compounds. Interestingly, the MYB72 interactor EIL3 (also called SLIM1 for SULFUR LIMITATION 1) was recently identified as a transcriptional regulator of sulfur limitation-induced signaling in *Arabidopsis* (Maruyama-Nakashita *et al.*, 2006). In addition, recent evidence has shown that nutrient availability has a profound effect on the expression of *MYB72*: low availability of iron, as well as toxic amounts of zinc cause *MYB72* expression in the roots (Colangelo & Guerinot, 2004; Van de Mortel *et al.*, 2006). Moreover, these conditions triggered excessive root-growth inhibition in *myb72* mutants in comparison to wild-type plants (Van de Mortel *et al.*, unpublished results), indicating that MYB72 alleviates nutrient stress conditions. Excess zinc is known to distort iron uptake by the plant, thereby mimicking the iron-limiting conditions that activate *MYB72* (Thomine *et al.*, 2003; Van de Mortel *et al.*, 2006). Together, these data indicate that enhanced *MYB72* expression is required to adequately respond to iron limitation. Fluorescent *Pseudomonas* spp., such as WCS417r, have the ability to efficiently take up iron under limiting conditions through the production of high-affinity iron-binding siderophores (Bakker *et al.*, 2007). In this perspective, it is tempting to speculate that the WCS417r-induced expression of *MYB72* is caused by iron deficiency. Further support for this comes from observations that both iron deprivation (Connolly *et al.*, 2003) and bacterization by WCS417r (Verhagen *et al.*, 2004) lead to induction of *FRO2 (FERRIC REDUCTION OXIDASE 2)* (Robinson *et al.*, 1999), which encodes a protein that is critical for iron uptake by the roots. However, it should be noted that

experiments performed in radish indicate that iron-limitation itself is not sufficient to induce systemic resistance against *Fusarium* wilt (Leeman *et al.*, 1996). Moreover, despite high levels of root colonization, not all *Pseudomonas* strains trigger ISR in all plant species (Van Loon & Bakker, 2006; Bakker *et al.*, 2007). Therefore, besides iron limitation at least one strain specific component is required for the induction of systemic resistance.

LONG-DISTANCE TRANSDUCTION OF RESISTANCE-INDUCING SIGNALS

Although SAR and ISR are physiologically different phenomena, both require the production and transport of a signaling molecule to enhance resistance in distal plant parts. In the case of SAR, SA was initially considered to be a likely candidate for the long-distance SAR signal, because levels of this phytohormone were found to rapidly increase locally upon pathogen infection, followed by a systemic increase (Métraux *et al.*, 1990). Further support for this hypothesis came from labeling studies that undisputedly demonstrated that at least part of the SA is transported from pathogen-infected leaves to uninfected SAR-expressing leaves of tobacco or cucumber (Shulaev *et al.*, 1995; Molders *et al.*, 1996). However, grafting experiments revealed that SA-degrading NahG rootstocks of tobacco are still capable of generating a SAR signal (Vernooij *et al.*, 1994), suggesting that SA itself is not the critical long-distance signal of SAR. Seskar *et al.* (1998) proposed methyl salicylate (MeSA) as a candidate for the systemic signal, as being synthesized from SA in the locally infected leaves and reconverted to SA in the systemic target tissues. Later findings that SAMT (SA METHYL TRANSFERASE) and the MeSA esterase SABP2 (SA-BINDING PROTEIN 2) are essential for the expression of SAR in locally infected and systemic leaves, respectively, are in exact agreement with this hypothesis (Kumar & Klessig, 2003; Forouhar *et al.*, 2005; Park *et al.*, 2007). Hence, in tobacco, MeSA is a critical long-distance SAR signal.

In *Arabidopsis* the necessity for MeSA in SAR has not been demonstrated yet. In contrast to findings by Park *et al*. (2007) in tobacco, Truman *et al*. (2007) found indications that JAs function as long-distance SAR signals in *Arabidopsis*. Transcriptome analysis of systemic tissues four hours after localized attack with an avirulent strain of *Pst* DC3000 indicated a large overlap with the expression profile of leaves responding to wounding or local herbivory. This transcriptional reprogramming coincided with a rapid increase of JA, and not SA, in petiolar exudates from pathogen-infected leaves. Furthermore, both the JA-biosynthesis mutant *opr3* and the JA-response mutant *jin1* (disrupted in the *MYC2* gene) were unable to generate SAR against *P. syringae* pv. *maculicola* and *Pst* DC3000. However, other mutants with a defect in JA-signaling, i.e. *jar1* and *eds8*, were previously found to express wild-type levels of SAR (Pieterse *et al.*, 1998; Ton *et al.*, 2002a). Therefore, the exact role of JAs in SAR signal transduction

requires further study. It is, nevertheless, noteworthy that lipid-derived compounds have been implicated in long-distance SAR signaling previously. In a screen for mutants defective in biologically-induced SAR against *P. syringae* pv. *tomato* and *H. parasitica*, Maldonado *et al*. (2002) identified *dir1* (*defective in induced resistance 1*). In contrast to petiolar exudates from pathogen-infected wild-type leaves, exudates from pathogen-infected *dir1* leaves failed to trigger *PR-1* expression upon pressure infiltration in *Arabidopsis* wild-type leaves. Remarkably, *dir1* was not affected in systemic induction of SA production after infection with an avirulent strain of *Pst* DC3000, suggesting that *Arabidopsis*, in addition to SA, requires a DIR1-dependent signal to express SAR. As *DIR1* encodes a lipid transfer protein, the DIR1 protein might interact with a lipid-derived molecule to mediate long-distance signaling. Further evidence supporting a role of lipid-derived signals in long-distance SAR signaling came from Nandi *et al*. (2004), who reported that the *Arabidopsis sfd1* mutant, which is affected in glycolipid synthesis, failed to transmit the SAR signal from infected to un-infected leaves.

The systemically transported long-distance signal of ISR remains to be identified. However, it seems plausible that the ISR long-distance signal, unlike that of SAR, is transported through the xylem, because this tissue provides a more conducive transportation pathway from root to shoot (Van Bel & Gaupels, 2004). It should, however, also be noted that leaf-infiltration with WCS417r renders distal plant parts more resistant against subsequent attack by *Pst* DC3000 (Pieterse *et al.*, 1996; 2000). Therefore, transport through the xylem may not be the only transportation pathway of the long-distance ISR signal. Similarly to SAR (Verberne *et al.*, 2003), ET might be involved in the generation of the systemic signal of ISR. Knoester *et al.* (1999) demonstrated that the *eir1* (*ethylene insensitive root 1*) mutant (also known as *agr* (Utsuno *et al.*, 1998) and *pin2* (Müller *et al.*, 1998)), of which only the root tissue is insensitive to ET, was not able to generate ISR to *Pst* DC3000 upon root colonization by WCS417r. Therefore, the authors concluded that for WCS417r-ISR responsiveness to ET is required, not only in the systemic plant tissues, but also locally, at the site of root colonization. What should be noted however, is that the EIR1 protein is not only obligatory for ET-signaling, but also for the responsiveness to internally generated auxin (AUX) (Luschnig *et al.*, 1998). For that reason, based on the results with the *eir1* mutant, AUX transport rather than ET signaling can not be excluded to be required in the local onset of ISR.

REGULATION OF PRIMING

Upon arrival of the systemically transported signal, the distal plant parts become more resistant to pathogen attack. In case of ISR, the enhanced resistance does not result from direct activation of defense mechanisms, but rather from priming for defense. When primed, the host plant is able to respond more effectively to pathogen attack. Defense reactions are activated faster and more strongly, leaving less opportunity for the attacker

to successfully infect the plant. It is commonly assumed that priming enhances the signaling capacity of the induced defense reaction. In theory, this happens at different steps in the signaling cascade ranging from the very early recognition of the pathogen, to the down-stream secretion of antimicrobial proteins and secondary metabolites. Amplification of the capacity of the rate-limiting bottleneck in signal-transduction will result in an increased capacity of the innate immune reaction.

TRANSCRIPTION FACTORS

By comparing the full-genome sequences of different eukaryotes, Riechmann *et al.* (2000) observed that an exceptionally large part of the *Arabidopsis* genome is dedicated to transcriptional regulation. Apparently, plants regulate their activity more through differential gene expression than other eukaryotes. In the promoter regions *cis*-acting elements serve as docking sites for transcription factors (TFs). By binding to these *cis*-acting elements, TFs can have either inducing or repressing effects on the transcriptional activity of the downstream gene. In this regard, an enhanced availability of defense-related TFs can easily lead to an increase in the over-all signaling capacity, either by directly enhancing the capacity for gene transcription, or through a transcriptional repression of negative regulators of the plant's induced defense reaction.

Transcription factor MYC2

Whole-genome transcriptome analysis of *Arabidopsis* leaf rosettes revealed that expression of 442 out of 1879 MeJA-responsive genes was primed by WCS417r bacteria (Chapter 4). Promoter analysis of these primed genes identified an enrichment for the G-box-related motif CACATG (Chapter 4). This motif is one of the *cis*-acting elements that can bind to the transcriptional regulator *MYC2* (Abe *et al.*, 1997; De Pater *et al.*, 1997). The CACATG motif was also enriched in the promoter regions of the ISR-primed genes of plants that showed an augmented response to attack by *Pst* DC3000 (Chapter 4; Verhagen *et al.*, 2004). Together, these data suggested a regulatory role for MYC2 in the priming for defense during WCS417r-ISR. Mutants disrupted in the *MYC2* gene (*jin1-1* and *jin1-2*) were tested for their ability to express WCS417r-ISR (Chapter 4). In contrast to wild-type Col-0, WCS417r-induced *jin1-1* and *jin1-2* plants did not show a reduction in bacterial speck disease after inoculation with *Pst* DC3000. Similarly, *jin1-2* failed to develop ISR against *H. parasitica*. Hence, MYC2 can be considered as an essential regulator for WCS417r-ISR.

To test whether root colonization by WCS417r leads to a systemic induction of *MYC2* gene expression, we analyzed *MYC2* transcripts in the leaves of non-induced and WCS417r-induced plants using Q-PCR (Chapter 4). Indeed, a higher amount of *MYC2* mRNA was detected in the WCS417r-treated plants compared to the non-induced control-treated plants. Thus, root colonization by WCS417r leads to a direct transcriptional induction of the *MYC2* gene. Previously, Verhagen *et al.* (2004) were

unable to detect induction of *MYC2* by WCS417r, because no probe sets for this gene were present on the 8k Affymetrix AG-arrays.

Transcription-factor profiling

The identification of MYC2 as an essential regulator of ISR prompted us to investigate whether also other TF genes show a direct transcriptional response in the leaves upon bacterization treatment of the roots. To this end, robotized Q-PCR was used to profile the level of transcription of all putative TF genes in the *Arabidopsis* genome in plants expressing WCS417r-ISR. A similar profiling was performed on leaves of BABA-treated plants, as the signaling pathway underlying ISR partially overlaps with that of BABA-IR (Chapter 5). Both root colonization by WCS417r and application of BABA to the roots resulted in the induction of a large number of largely non-overlapping TFs in the leaves (Chapter 5). The set of TFs induced by WCS417r (90), was enriched in *AP2/ERFs* (17), which have been implicated in the regulation of JA- and ET-regulated responses (Solano *et al.*, 1998; Park *et al.*, 2001; McGrath *et al.*, 2005). This finding fits nicely with earlier observations that the majority of genes with a primed induction after *Pst* DC3000 challenge are JA/ET-responsive (Verhagen *et al.*, 2004).

Notably, ERF1 was one of the TFs that showed an enhanced expression in the leaves upon colonization of the roots by WCS417r. This TF has been demonstrated to function in mutually antagonistic action with MYC2 in the regulation of, on the one hand, the wound response, and on the other hand, defense responses against pathogens (Lorenzo *et al.*, 2004; Lorenzo & Solano, 2005). However, during priming for defense this antagonism might not even occur. The induction of TFs does not result in direct activation of defense responses. In order to do so, a second, stress-related signal is required. In analogy, this secondary signal might also be required to activate the antagonistic action between ERF1 and MYC2. Therefore, accumulation of mRNAs of both TFs might increase responsiveness of both the wounding response, as well as the response against microbial pathogens, depending on the nature of the secondary signal. In line with this hypothesis, ISR has been demonstrated to be effective against necrotrophic pathogens (Chapter 2; Ton *et al.*, 2002b) and insects (Van Oosten, 2007). Furthermore, colonization of Arabidopsis roots primes the expression of JA-responsive genes, such as *LOX2* and *VSP2* (Chapters 4 & 5; Van Wees *et al.*, 1999), but also that of *PDF1.2* (Van Oosten, 2007), which is regulated by JA and ET.

Application of low amounts of BABA to the roots of *Arabidopsis* induced the expression of 186 TFs in the leaves (Chapter 5). Among these, expression of 21 out of the total of 71 *WRKY* TFs was increased. WRKY TFs have been implicated to co-regulate the transcription of several defense-related genes (Dong *et al.*, 2003). Moreover, some of these were identified to act downstream of NPR1 during SAR (Wang *et al.*, 2006). Because BABA-IR against *Pst* DC3000, like SAR, depends on NPR1 (Zimmerli *et al.*, 2000; Kohler *et al.*, 2002), the effect of BABA application on *WRKY* expression was also investigated in *npr1* mutant plants (Chapter 5). Strikingly, 20 out of the 21 BABA-

responsive *WRKYs* in wild-type plants were not responsive to BABA in the *npr1* mutant. Together, these findings strongly suggest that WRKY TFs are involved in regulation of the SA/NPR1-dependent branch of BABA-IR, but not in WCS417r-ISR.

Promoter analysis of TF-genes

To elucidate potential factors upstream of the observed TF gene induction in the signaling pathway of WCS417r-and BABA-induced priming, we examined the promoter regions of the different sets of TFs for enrichment of specific *cis*-acting elements (Chapter 5). Both the WCS417r-inducible and the BABA-inducible TF genes showed an overrepresentation of the G-box and the PLGT1-box, which have been associated to responses to pathogen infection and salt stress (Dröge-Laser *et al.*, 1997; Faktor *et al.*, 1997; Boter *et al.*, 2004; Park *et al.*, 2004). On the other hand, promoters of BABA-responsive TFs were significantly enriched in W-box elements, which function as binding sites for WRKY TFs. In combination with our observation that many WRKY genes are directly induced by BABA, this enrichment of W-boxes in BABA-inducible TF gene promoters points to a signaling amplification during the onset of BABA-induced priming. In a first step to identify early signaling components preceding the WRKY-dependent signaling amplification, the promoter regions of the BABA-responsive WRKYs were compared to the BABA-non-responsive ones. This analysis revealed a very significant over-representation of a yet uncharacterized TAG[TA]CT motif in the promoters of the NPR1-dependent, BABA-inducible *WRKY* genes. It is tempting to speculate that this motif acts as a binding site for a master-switch regulator in the BABA-induced priming of SA-inducible defense. Perception of BABA would lead to a very rapid activation of this factor, causing TAG[TA]CT-dependent activation of WRKY genes. Subsequently, the induced WRKYs would activate other TF genes resulting in an enhanced number of many different defense-related TFs. This primed alarm state would facilitate a faster and stronger induction of defense-related genes upon pathogen attack.

Activation of transcription factors

Although expression of TF genes is directly induced by WCS417r bacteria or BABA (Chapters 4 & 5), downstream activation of defense-related genes is not. Therefore, besides the presumed accumulation of TF proteins, a second pathogen-derived signal is required to activate, or lift repression of these TFs upon pathogen infection. There is ample evidence that TFs require post-translational activation in order to exert their activity.

Previously, Menke *et al.* (2004) demonstrated that silencing of *MPK6* (*MITOGEN ACTIVATED KINASE 6*) compromised both *R*-mediated resistance and basal resistance of *Arabidopsis* against *P. syringae* pv. *tomato* and *H. parasitica*, indicating that MAPK-signaling cascades are involved in disease resistance. Moreover, recent results by Beckers *et al.* (2007) showed that both MPK3 and MPK6 are involved in the priming for

defense that occurs during chemically-induced resistance. Application of the priming agent BTH (benzothiadiazole) resulted in the accumulation of these proteins. However, phosphorylation and enzyme activity of MPK3 and MPK6 only occurred upon a secondary stress treatment. During ISR and BABA-IR, similar MAPK components might act upstream of the accumulated TFs and activate the latter after detection of a stress signal.

Recent insights on the functioning of nuclear inner membrane (NIM) proteins of mammalian cells provide an alternative mechanisms by which TFs are kept inactive. These insights indicate that NIM-proteins sequester TFs to the NIM and thereby limit or completely repress their activities (Heessen & Fornerod, 2007). If the enhanced sequestering of TFs to the NIM also occurs in cells of primed plants, stress-initiated release would result in the sudden presence of active TFs in the nucleus.

OTHER REGULATORY MECHANISMS OF PRIMING

In this study, we established a clear association between priming for defense and enhanced expression of TFs (Chapters 4 & 5). However, this finding does not exclude involvement of additional layers of regulation. As mentioned above, Beckers *et al*. (2007) identified MPK3 and MPK6 as regulators of priming during SAR. Moreover, recent results point to possible involvement of epigenetic regulation in BABA-induced priming (Jurriaan Ton, unpublished results). Chromatin immunoprecipation (ChIP) revealed that BABA induces enhanced association of the *PR-1* promoter to the acetylated form of histone 3 (H3), which allows for a more open chromatin structure (Pfluger & Wagner, 2007). Interestingly, this BABA-induced acetylation of H3 was still present in the *npr1* mutant, indicating that BABA-induced chromatin remodeling does not require NPR1. Moreover, this implies that enhanced H3 acetylation around the *PR-1* promoter is not caused by direct transcriptional induction of *PR-1*, but results from a yet unknown signal that primes the *PR-1* promoter for enhanced binding to WRKY and TGA TFs.

Protein folding and secretion

Also at the post-transcriptional level, physiological changes in cell organization can contribute to the enhanced defensive capacity during priming. Correctly-folded proteins rather than mRNA molecules are the active players in defense. To be effective, these proteins often need to be secreted to specific sites in the cell or apoplast. Therefore, enhancing the output of these mechanisms will positively contribute to the priming of defense responses. Protein folding can be accelerated by higher amounts of folding chaperones. Transcription rates of some of these chaperones, such as *BIP2* (*LUMINAL-BINDING PROTEIN 2*), have been reported to be up-regulated in a NPR1-dependent manner upon activation of chemically-induced resistance (Wang *et al.*, 2005). Moreover, the NPR1-dependent expression of *BIP2* and other genes that regulate protein folding and

secretion, is differently regulated by NPR1 than the expression of defense-related genes, such as *PR-1* (Wang *et al.*, 2005).

Although ISR, like SAR, requires NPR1 (Pieterse *et al.*, 1998), it is not associated with a direct induction or priming of genes encoding for SA-inducible PR-proteins (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Therefore, it is not unlikely that the regulation of the protein secretory pathway is the shared NPR1-dependent component between the SAR and ISR pathway. In line with this hypothesis, preliminary data demonstrate that mutants disrupted in NPR1-regulated secretory genes (e.g. *bip2*, and *sec61*) lost their ability to express WCS417r-ISR against *Pst* DC3000 and *H. parasitica* (Van der Ent *et al.*, unpublished results). Furthermore, in contrast to the situation in wild-type *Arabidopsis* plants, WCS417r failed to prime *npr1* for enhanced deposition of callose-containing cell wall appositions at sites of attempted *H. parasitica* entry (Chapter 5). This not only demonstrates that NPR1 is required for the enhanced deposition of callose and other components of the papillae, but also again links NPR1 to the protein secretory pathway, as the latter has been related to the formation of cell-wall appositions (Staiger, 2000; Hardham, 2007). What should be noted, is that the enhanced formation of callose-containing cell-wall appositions that occurs during BABA-IR is independent of NPR1 (Chapter 5; Zimmerli *et al.*, 2000). This indicates that at least one other signaling cascade can result in the enhanced responsiveness of this defense mechanism.

Previously, the IBS2/SAC1b and the IBS3/ABA1 proteins have also been related to augmentation of callose deposition (Ton *et al.*, 2005). IBS2 shows homology to yeast polyphosphoinositide phosphatase SAC1 (Guo *et al.*, 1999; Despres *et al.*, 2003b). In yeast, disruption of the SAC1 gene affected protein secretion and organization of the cytoskeleton, due to an accumulation of substrates for SAC1 (Hama *et al.*, 1999; Caroni, 2001; Foti *et al.*, 2001; Schorr *et al.*, 2001). Most likely, IBS2 serves a similar function in plants, as yeast SAC1 was demonstrated to complement *ibs2/sac1b* null-mutants. These observations again form a link between the formation of cell-wall appositions and the secretory system. The *ibs3* mutant was shown to contain a T-DNA insertion in the *IBS3/ABA1* gene (Ton *et al.*, 2005). Although the role of ABA in abiotic stress adaption is well established (Zhu, 2002), its contribution to disease resistance is contentious (Flors *et al.*, 2005). Several recent reports, however, have pinpointed a positive relation between ABA and callose depositions at sites of attempted pathogen entry in barley, *Arabidopsis* and tomato (Ton & Mauch-Mani, 2004; Wiese *et al.*, 2004; Flors *et al.*, 2005; Asselbergh & Höfte, 2007). The strongest causal link between the two phenomena was provided by the fact that ABA-insensitive *abi4-1* mutants of *Arabidopsis* were disrupted in their ability to show a BABA-triggered augmented callose deposition at sites of attempted penetration by *P. cucumerina* or *A. brassicicola* (Ton & Mauch-Mani, 2004). Moreover, exogenous application of ABA to wild-type *Arabidopsis* mimicked the effect of BABA on both callose deposition and resistance against the two necrotrophic pathogens.

Notably, both *ibs2* and *ibs3* show normal basal levels of callose deposition, but are disrupted in the BABA-induced and WCS417r-induced augmentation of this response (Chapter 5; Ton *et al.*, 2005). Most likely, priming of other defense responses is not

affected in *ibs2* and *ibs3*, as these mutants are still able to generate BABA-IR and WCS417r-ISR against *Pst* DC3000 (Chapter 5; Ton *et al.*, 2005).

In conclusion, the results denoted in this thesis demonstrate that priming agents are able to improve the effectiveness of the plant's innate immune system by modifications at different cellular levels ranging from gene-expression to protein secretion. The defense mechanisms that become primed and subsequently activated depend on the priming-inducing agent and the attacker, respectively.

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SUMMARY

To defend themselves against pathogen or insect attack, plants can activate a wide array of inducible defense mechanisms. When plants are in a state of induced resistance, the responsiveness and / or capacity of these inducible defenses are enhanced. A classic example of this induced resistance is systemic acquired resistance (SAR) that is triggered after infection by a necrotizing pathogen and renders uninfected, distal plant parts more resistant to subsequent pathogen attack. A physiologically distinct type of induced resistance is triggered after colonization of the roots of plants by selected strains of non-pathogenic rhizobacteria. In *Arabidopsis thaliana,* this induced systemic resistance (ISR) is regulated by a jamonic acid (JA) and ethylene (ET)-dependent signaling pathway. In contrast to SAR, rhizobacteria-mediated ISR is not associated with an increase in the expression of genes encoding pathogenesis-related proteins.

Previously, microarray analysis revealed that colonization of *Arabidopsis* roots by ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria alters the expression of a large number of genes in the roots. One of these root-specific, WCS417r-induced genes codes for the R2R3 MYB-domain containing transcription factor (TF) protein MYB72. Two independent knockout mutants harboring a T-DNA insertion in the *MYB72* gene (*myb72-1* and *myb72-2*) were blocked in their ability to mount ISR upon root colonization by WCS417r or the ISR-inducing strain *Pseudomonas putida* WCS358r (Chapter 2). This block in ISR induction was not only apparent against the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, but also against the oomycete pathogen *Hyaloperonospora parasitica* and the necrotrophic fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea*. These data demonstrate that *MYB72* is required for ISR-signaling. However, constitutive *MYB72* expressers did not show a constitutively enhanced levels of resistance, suggesting that MYB72 is not sufficient to trigger ISR. Therefore, activation of at least one other component of the ISR signal-transduction cascade is co-required. Yeasts two-hybrid experiments demonstrated a physical interaction of MYB72 with the EIN3-like protein EIL3 *in vitro*. The latter has been implicated in the regulation of responses to ET and is as such a likely candidate involved in MYB72-dependent ISR signaling.

Besides non-pathogenic rhizobacteria, such as *Pseudomonas spp*., selected isolates of non-pathogenic soil-borne fungi are also know to enhance the plant's defensive capacity. *Trichoderma spp.* have been demonstrated to induce systemic disease resistance in several plant species. However, the underlying signaling cascades are poorly understood... In *Arabidopsis* wild-type Col-0 and the SAR-compromised mutant *sid2*, *Trichoderma asperellum* strain T34 induced an enhanced level of protection against *P. syringae* pv. *tomato*, *H. parasitica* and *Plectosphaerella cucumerina,* whereas this protection was absent in the ISR mutant *myb72*. These results indicate that *T. asperellum* T34-induced resistance resembles rhizobacteria-mediated ISR rather than pathogen-induced SAR (Chapter 3).

Although microarray analyses revealed that colonization of *Arabidopsis* roots by ISR-inducing WCS417r leads to local changes in transcriptional activity, in the aboveground tissues no direct changes in gene-expression could be detected. However, after challenge inoculation, a large set of pathogen-responsive genes showed an augmented expression pattern, indicating that the expression of ISR is associated with priming of pathogen-responsive gene expression. The majority of these primed genes are regulated by a JA- and/or ET-dependent signaling pathway. To investigate the molecular mechanism underlying this priming phenomenon, we monitored the expression of all methyl JA (MeJA)-responsive genes in control and WCS417r-ISR-expressing plants using Affymetrix Arabidopsis ATH1 whole-genome GeneChips (Chapter 4). Promoter analysis of the genes that showed a WCS417r-primed response to MeJA, revealed a significant enrichment for the CACATG motif. The CACATG motif was previously demonstrated to serve as a docking site for the TF MYC2, which is involved in the regulation ABA and JA responses. Mutants of *Arabidopsis* that carry a defect in the *MYC2* gene (*jin1-1* and *jin1-2*), were not able to express WCS417r-ISR against *P. syringae* pv. *tomato* and *H. parasitica*, indicating that MYC2 plays an important role in priming for enhanced JA-responsive gene expressing during WCS417r-ISR.

In order to gain insight in the molecular mechanisms underlying priming for enhanced defense, we performed a comparative study between WCS417r-ISR and resistance induced by the non-protein amino acid ß-aminobutyric acid (BABA-IR). Both WCS417r-ISR and BABA-IR are based on priming for enhanced defense, rather than on direct activation of defense. Like BABA-IR, WCS417r-IR against *H. parasitica* was shown to be associated with priming for enhanced formation of callose-containing cell-wall appositions at sites of pathogen entry. Mutants with a disruption in the *IBS2* or *IBS3* gene were specifically blocked in their ability to show this enhanced callose-deposition and consequently were affected in both BABA-IR and WCS417r-ISR. This indicates that the BABA-IR and WCS417r-ISR pathways share signaling components involved in priming for enhanced callose deposition (Chapter 5).

Being important regulators of gene expression, TFs might also take part in the regulation of priming for defense. An increased pool of inactive TFs would not lead to direct transcriptional changes, but would enhance the response upon subsequent activation by a stress signal. Q-PCR-based expression profiling of all putative *Arabidopsis* TFs indeed indicated an higher transcriptional activity of a subset of TF-encoding genes in the systemic tissue of WCS417r- and BABA-primed plants (Chapter 5). The set of WCS417r-induced TF-genes was enriched for those involved in the regulation of responses to JA, such as AP2/ERFs and MYC2. Amongst the TF genes of which the expression was induced by BABA, 21 encoded WRKYs, which have been demonstrated to regulate downstream processes in SA-signaling. Thus, induced resistance is accompanied by a systemic increase in the activity of a subset of TF genes, the composition of which depends on the nature of the priming agent.

Collectively, the work described in this thesis advanced our understanding of rhizobacteria-mediated ISR and provided novel insights in the molecular mechanisms

underlying priming for enhanced defense. Priming of pathogen-responsive genes allows the plant to react more effectively to the invader encountered, which might explain the broad-spectrum action of rhizobacteria-mediated ISR.

SAMENVATTING

Planten zijn in staat in hun eigen energiebehoeften te voorzien. Gedurende het proces van fotosynthese gebruiken planten de energie van ingevangen fotonen om energierijke koolstofverbindingen (suikers) te maken. Deze suikers kunnen wanneer en waar nodig worden verbrand om de hierbij vrij komende energie te gebruiken om alle vereiste processen te laten verlopen. De meeste andere organismen, waaronder vele bacterie- en schimmelsoorten, maar ook mensen en andere dieren, zijn eveneens afhankelijk van de door planten gevormde suikers om in hun energiebehoeften te kunnen voorzien. Vandaar dat planten continu staan blootgesteld aan aanvallen van microbiële ziekteverwekkers en aan vraat door insecten en andere diersoorten.

Om zich tegen hun belagers te verweren beschikken planten over verschillende afweermechanismen. Sommige hiervan zijn continu aanwezig en verhinderen of beperken de aanvaller in het bereiken van de inhoud van de plantencellen. Anderen worden alleen geproduceerd als reaktie op de belager. Voorbeelden hiervan zijn zogenaamde fytoalexinen, proteïnase inhibitoren en 'pathogenesis-related proteins' (PR-eiwitten). Tezamen voorkomen of vertragen de constitutieve en induceerbare afweermechanismen de ziekteontwikkeling en vormen de basisresistentie van de plant.

Planten zijn in staat om het basisniveau van resistentie tegen toekomstige aanvallen te verhogen, een fenomeen dat bekend staat als geïnduceerde resistentie. Zo leidt een infectie door een pathogeen niet alleen tot de lokale inductie van afweermechanismen, maar eveneens tot een systemische toename van de afweercapaciteit in alle plantendelen. Deze systemische verworven resistentie (systemic acquired resistance; SAR) is niet alleen effectief tegen de primaire ziekteverwekker, maar ook tegen een groot aantal andere typen pathogenen. Een resistentie die fenotypisch vergelijkbaar is met SAR wordt geïnduceerd na kolonisatie van de wortels door bepaalde niet-ziekteverwekkende *Pseudomonas* soorten. Deze vorm van geïnduceerde resistentie wordt ook wel induceerbare systemische resistentie genoemd (ISR). Hoewel ISR net als SAR gepaard gaat met het verhogen van de afweercapaciteit van de gehele plant, bestaan er verschillen tussen deze twee vormen van geïnduceerde resistentie. Bij de het activeren van SAR speelt het plantenhormoon salicylzuur (salicylic acid; SA) een belangrijke rol, terwijl voor ISR juist gevoeligheid voor de hormonen ethyleen (ET) en jasmonzuur (jasmonic acid; JA) is vereist. Het onderzoek beschreven in dit proefschrift had als doel om de moleculaire mechanismen van ISR te onderzoeken.

Een inventarisatie van de genexpressie in *Arabidopsis thaliana* liet zien dat een groot aantal *Arabidopsis* genen reageren op kolonisatie van de wortels door de ISR-inducerende stam *Pseudomonas fluorescens* WCS417r. Een van de wortel-specifieke WCS417r-responsieve genen codeert voor de transcriptie factor (TF) MYB72. Twee onafhankelijke *myb72* mutanten van *Arabidopsis* waren niet langer in staat ISR te genereren wanneer hun wortels waren gekoloniseerd door WCS417r of door een andere ISR-inducerende stam *Pseudomonas putida* WCS358r (Hoofdstuk 2). Deze resultaten

toonden aan dat de MYB72 TF een belangrijke schakel is in ISR signaaltransductieroute. Echter, MYB72 alleen was niet afdoende om de afweercapaciteit van de plant te verhogen. *MYB72* overexpressors verschilden niet van wild type *Arabidopsis* planten in hun resistentie tegen verschillende pathogenen. Waarschijnlijk is dus naast expressie van *MYB72* minimaal één andere signaal-transductie-component nodig om ISR te activeren. Experimenten in gist toonden aan dat MYB72 kan interacteren met EIL3, een homoloog van de TF EIN3 welke belangrijk is in de ET signaaltransductieroute. Vervolgonderzoek is nodig om uit te zoeken wat de rol van de interactie tussen MYB72 en EIL3 is in ISR.

Niet-pathogene bodemschimmels zoals *Trichoderma spp.* kunnen ook de afweercapaciteit van de plant verhogen. Echter, de signaaltransductieroute die ten grondslag ligt aan *Trichoderma*-geïnduceerde resistentie is grotendeels onbekend. *Trichoderma asperellum* T34 was in staat de afweer tegen *Pseudomonas syringae* pv. *tomato*, *Hyaloperonospora parasitica* en *Plectosphaerella cucumerina* te verhogen in het *Arabidopsis* wild-type Col-0 en in de SAR-mutant *sid2*, maar niet in de ISR-mutant *myb72* (Hoofdstuk 3). Deze resultaten duiden aan dat T34-geïnduceerde resistentie via de ISR signaaltransductieroute verloopt.

Hoewel kolonisatie van *Arabidopsis* wortels door WCS417r lokaal de expressie van vele genen beïnvloedt, brengt het systemisch geen directe veranderingen in genexpressie teweeg. Wel leidt wortelkolonisatie door WCS417r ertoe dat een grote groep genen sneller en / of heftiger reageert op blootstelling aan de bacteriële ziekteverwekker *P. syringae* pv. *tomato*, hetgeen een indicatie is dat ISR geassocieerd is met "priming" van afweergerelateerde genexpressie. Het overgrote deel van deze geprimede genen wordt gereguleerd door JA. Om een beter inzicht te krijgen in het moleculaire netwerk dat ten grondslag ligt aan deze vorm van priming hebben we de expressiepatronen van alle door methyl-jasmonzuur (MeJA) gereguleerde genen in zowel controle als WCS417r-behandelde planten bestudeerd met behulp van Affymetrix *Arabidopsis* ATH1 GeneChips waarop het hele genoom van *Arabidopsis* is gerepresenteerd (Hoofdstuk 4). Sequentieanalyse van de promoterregio's van door WCS417r-geprimede genen bracht aan het licht dat deze verrijkt waren met het CACATG-motief. Uit eerdere studies bleek dat dit motief als bindingsplaats fungeert voor de TF MYC2, een regulator van ABA- en JA-gestuurde processen. *Arabidopsis* mutanten met een defect in het *MYC2* gen (*jin1-1* en *jin1-2*) waren niet in staat ISR tot expressie te brengen tegen *P. syringae* pv. *tomato* of *H. parasitica*. Deze resultaten laten zien dat de TF MYC2 een belangrijke rol speelt in ISR, mogelijk als transcriptionele regulator van door WCS417r-geprimede genen.

Om de regulatie van priming verder te bestuderen, hebben we WCS417r-ISR vergeleken met resistentie zoals dat wordt geïnduceerd door het aminozuur ß-aminoboterzuur (ß-aminobutyric acid; BABA). Zowel WCS417r-ISR als door BABA geïnduceerde resistentie (BABA-IR) is gebaseerd op priming voor een versnelde vorming van callose-bevattende celwandverstevigingen op plaatsen waar schimmels en oomyceten de plant trachten binnen te dringen. De *Arabidopsis* mutanten *ibs2* en *ibs3* zijn verstoord in deze vorm van priming en bleken ook niet in staat om WCS417r-ISR en BABA-IR tot expressie te brengen tegen *H. parasitica*. Uit deze resultaten kan

geconcludeerd worden dat de WCS417r-ISR en BABA-IR signaaltransductieroutes gedeeltelijk overlappen voor wat betreft priming voor pathogeen-geïnduceerde callosevorming (Hoofdstuk 5).

Zowel WCS417r-ISR als BABA-IR zijn gekarakteriseerd door priming. Geprimede planten vertonen een versterkte expressie van afweergenen na aanval door een pathogeen. Transcriptiefactoren spelen een belangrijke rol bij de regulatie van genexpressie. Om de rol van transcriptiefactoren in priming te onderzoeken is met behulp van Q-PCR de expressie van alle 2300 TF genen van *Arabidopsis* geanaliseerd in planten die WCS417r-ISR en BABA-IR tot expressie brachten. Zowel WCS417r-ISR als BABA-IR bleek gepaard te gaan met de activatie van een groot aantal TF genen (Hoofdstuk 5). De set van door WCS417r geïnduceerde genen bevatte een relatief groot aantal AP2/ERF TFs waarvan vele eerder in verband zijn gebracht met de respons van planten op JA. Onder de door BABA geïnduceerde TF genen bevonden zich 21 *WRKY*s, waarvan is aangetoond dat ze een regulerende rol spelen in door SA gestuurde processen. De resultaten uit deze studie laten zien dat WCS417r-ISR en BABA-IR gepaard gaan met een verhoogde expressie van een groot aantal TF genen. Van zichzelf doen deze transcriptiefactoren nog niet veel. Echter, wanneer een geprimede plant wordt aangevallen, worden de transcriptiefactoren geactiveerd en beschikt de plant over een verhoogde afweercapaciteit waardoor hij beter in staat is om zich succesvol te verdedigen tegen zijn vijandelijke belagers.

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Pieterse, C.M.J., Van der Ent, S., Van Pelt, J.A., & Van Loon, L.C. (2007). The role of ethylene in rhizobacteria-induced systemic resistance (ISR). In: Advances in Plant Ethylene Research: Proceedings of the 7th International Symposium on the Plant Hormone Ethylene (Ramira, A., Chang, C., Giovannoni, J., Klee, H., Perata, P., and Woltering, E., eds), Springer, Dordrecht, the Netherlands, pp. 325-331.

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CURRICULUM VITAE

Sjoerd van der Ent werd geboren op 28 december 1979 te Rotterdam. Onder de rook van deze prachtige stad en het toeziend oog van beide liefhebbende ouders groeide hij op in de rustige bloemenbuurt van Krimpen aan den IJssel. In 1998 verliet hij na zes mooie jaren het in Capelle aan den IJssel gelegen Comenius College met een VWO diploma op zak en zijn toekomstige vrouw aan de hand. Een korte scheikundige start aan de Technische Universiteit Delft ten spijt werd in datzelfde jaar aangevangen met de studie Biologie aan de Universiteit Utrecht. Als inmiddels gelukkig getrouwd man vervulde hij in de doctoraalfase van deze opleiding tussen vele bijbaantjes door twee onderzoekstages: bij de projectgroep Fytopathologie van de Universiteit Utrecht verrichtte hij onder begeleiding van Mareike Viebahn onderzoek aan de zogenaamde 'supercolonizer' van tarwe wortels, *Pseudomonas fluorescens* Q8r1-96r; en bij het genetica laboratorium van de R&D afdeling van DSM Food Specialties werkte hij aan de verbetering van een productiestam onder begeleiding van Peter Dekker. In september 2003 studeerde hij af, om in de daaropvolgende maand voor vier jaar als AIO aan de slag te gaan bij de toenmalige projectgroep Fytopathologie van de Universiteit Utrecht, hetgeen inmiddels is omgedoopt tot de sectie Plant-Microbe Interactions. Daar werd onder de bezielende begeleiding van Prof.dr.ir. C.M.J. Pieterse, Prof.dr.ir. L.C. van Loon en Dr. J. Ton het onderzoek uitgevoerd dat in dit proefschrift is beschreven.

NEW

PLANT DEFENSE ON ITS MARKS
The new PRIMING component allows plants to respond quicker and more fiercely to attackers encountered. Pathogens and insects do not have the opportunity to cause damage to the plant!

ALLIED FORCES
A friend in need is a friend indeed: PLANT DEFENSE makes plants benefit from their biotic surroundings. PGPR and PGPF enhance the defensive capacity of systemic tissues, while VOC-based S.O.S.-signaling attracts enemies of attacking insects.

COST-EFFECTIVE ENERGY TECHNOLOGY
PLANT DEFENSE provides major defensive capacity, without squandering precious assimilation-derived energy. Phytohormonal cross-talk and naturally occurring negative feedback loops do the trick!

INGREDIENTS:
Constitutive physical and chemical barriers, PRR-based PTI (including non-host resistance), R-gene-mediated ETI, and priming-based induced resistances (SAR, PGPR- and PGPF-mediated ISR, WIR and BABA-IR). Immunity and resistance is based on activation of one or more of the following defenses: formation of cell-wall appositions; lignification; production of phenolic compounds, reactive oxygen species, camalexin and/or other phytoalexins, PRs, PIs and other defense-related proteins; VOC-based S.O.S.-signaling to attract predators of enemies, and many more.

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