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Rhizobacteria-mediated induced systemic resistance in *Arabidopsis*

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Abstract: Selected strains of rhizosphere bacteria have been shown to reduce disease by activating a resistance mechanism in the plant called rhizobacteria-mediated induced systemic resistance (ISR). ISR resembles pathogen-induced systemic acquired resistance (SAR), in that both types of induced resistance render uninfected plant parts more resistant towards a broad spectrum of pathogens. The spectrum of effectiveness of ISR and SAR largely overlaps but is also partly divergent. In contrast to SAR, ISR induced by *Pseudomonas fluorescens* WCS417r is independent of salicylic acid (SA) and *PR* gene activation. Instead, ISR follows a signaling pathway in which components from the jasmonic acid (JA) and ethylene (ET) response are successively engaged to trigger a defense reaction that, like SAR, is controlled by the regulatory factor NPR1. To investigate the role of JA and ET in ISR, their production was monitored in ISR-expressing plants. Neither JA nor ET production changed upon induction of ISR. From this we postulate that ISR is mediated via an increase in the plants sensitivity to JA and ET. This is supported by the potentiated expression of the JA-inducible gene *AtVSP* observed in challenged, ISR-expressing plants. Moreover, preliminary results indicate that the ACC oxidase activity is enhanced in ISR-expressing plants, providing a greater potential to produce ET upon challenge. In our search for ISR-related genes we identified two genes that show altered expression upon induction of ISR: the JA-inducible gene *AtVSP*, which shows an enhanced level of expression in challenged, ISR-expressing plants, and a root-specific, ET-inducible thaumatin-like gene, which is activated upon colonization of the roots with ISR-inducing rhizobacteria. Moreover, we identified a locus (*ISR1*) on chromosome 3 that controls the expression of ISR. *Arabidopsis* genotypes that are affected in this locus are also less sensitive to ET. Together, these data confirm the important role of JA and ET in ISR signaling. Cross-talk between SA- and JA-dependent pathways can result in inhibition of JA-mediated defense responses. For instance, chemical agents that activate the SAR pathway, e.g. SA and benzothiadiazole (BTH), can affect the JA-dependent wound response, which plays a role in defense against insects. We investigated possible antagonistic interactions between the SAR pathway and the ISR pathway. Simultaneous activation of SAR and ISR in *Arabidopsis* resulted in an additive effect on the level of induced protection against *Pseudomonas syringae* pv. *tomato*. In *Arabidopsis* genotypes that are blocked in either SAR or ISR, this additive effect was not evident. Moreover, induction of ISR did not affect the expression of the SAR marker gene *PR-1* in plants expressing SAR. Together, these observations demonstrate that the SAR and the ISR pathway are compatible and that there is no significant cross-talk between these pathways. Therefore, combining SAR and ISR provides an attractive tool for the improvement of disease control.

Key words: cross-talk, defense signaling, ethylene, ISR, jasmonic acid, salicylic acid, SAR

Introduction

Plants possess several pathogen-inducible defense mechanisms that are active against microbial pathogens. A classic example of induced resistance is activated after primary infection with a necrotizing pathogen, rendering distant, uninfected plant parts more resistant towards a broad spectrum of pathogens (Kuc, 1982). This form of induced resistance is often

referred to as systemic acquired resistance (SAR; Ross, 1961; Ryals *et al.*, 1996; Sticher *et al.*, 1997), and has been demonstrated in many plant-pathogen interactions. Another form of induced disease resistance is triggered by selected strains of non-pathogenic rhizobacteria. Rhizosphere bacteria are present in large numbers on plant root surfaces, where root exudates and lysates provide nutrients. Certain strains of rhizosphere bacteria stimulate plant growth and are, therefore, called plant growth-promoting rhizobacteria. Strains that were isolated from naturally disease-suppressive soils, mainly fluorescent *Pseudomonas* spp., promoted plant growth by suppressing soil-borne pathogens. This biological control activity is effective under field conditions (Zehnder *et al.*, 2001) and in commercial greenhouses (Leeman *et al.*, 1995), and can be the result of competition for nutrients, siderophore-mediated competition for iron, antibiosis or the production of lytic enzymes (Bakker *et al.*, 1991). Some of these biological control strains are also able to reduce disease through a plant-mediated mechanism that is phenotypically similar to SAR, as the induced resistance is systemically activated and extends to above-ground plant parts. To facilitate distinguishing this type of induced resistance from pathogen-induced SAR, the term rhizobacteria-mediated induced systemic resistance (ISR) was introduced (Pieterse *et al.*, 1996; Van Loon *et al.*, 1998). Rhizobacteria-mediated ISR has been demonstrated in many plant species, e.g. bean, carnation, cucumber, radish, tobacco, and tomato, and has been reported to be effective against a broad spectrum of plant pathogens, including fungi, bacteria and viruses (Pieterse *et al.*, 2001a; Van Loon *et al.*, 1998). Previously, we developed an *Arabidopsis*-based model system to study the molecular basis underlying rhizobacteria-mediated ISR (Pieterse *et al.*, 1996). In this paper we will present the current state-of-the-art of the molecular basis of rhizobacteria-mediated ISR in *Arabidopsis*.

Material and methods

For experimental details see primary literature as cited in the text.

Results and discussion

Differential activation of rhizobacteria-mediated ISR in Arabidopsis

The ability to induce ISR in *Arabidopsis* was investigated using different ISR-inducing rhizobacterial strains and different *Arabidopsis* accessions. Colonization of the roots by ISR-inducing *P. fluorescens* WCS417r bacteria protected the plants against different types of pathogens, including the bacterial leaf pathogens *P. syringae* pv. *tomato* and the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani* (Pieterse *et al.*, 1996). Protection against these pathogens was typically manifested as both a reduction in disease symptoms and inhibition of pathogen growth. Since the rhizobacteria remained localized on the roots and thereby spatially separated from the challenging pathogen, it was concluded that the mode of action of disease suppression is through the activation of ISR in the plant.

Elicitation of ISR against *P. syringae* pv. *tomato* depended on the host/rhizobacterium combination. For instance, *Pseudomonas putida* WCS358r and *P. fluorescens* WCS374r performed differently on different plant species: *Arabidopsis* was responsive to WCS358r (Van Wees *et al.*, 1997), which is not effective in radish and carnation. Conversely, *Arabidopsis* was not responsive to WCS374r, a strain, which is a good inducer of ISR in radish. Also differential induction of ISR occurred between *Arabidopsis* accessions. Most accessions, e.g. Columbia and Landsberg *erecta*, were responsive to treatment with WCS417r, whereas accessions RLD and Wassilewskija were not (Ton *et al.*, 1999; Van Wees *et al.*,

1997). This suggests that specific recognition between the plant and the ISR-inducing rhizobacterium is required for the induction of ISR, and that ISR is genetically determined.

A novel signaling pathway controlling induced systemic resistance in Arabidopsis

The signaling pathway controlling pathogen-induced SAR has been well studied in *Arabidopsis*. As in many other species, SAR in *Arabidopsis* is dependent on SA and is tightly correlated with the activation of *PR* genes (Mauch-Mani and Métraux, 1998; Ryals *et al.*, 1996). To dissect the ISR signaling pathway in *Arabidopsis* we tested a large set of mutants that are impaired in their response to the defense signals SA, JA or ET (for overview see Table 1 in Pieterse *et al.* 2001a). In contrast to SAR, WCS417r-mediated ISR in *Arabidopsis* appeared to function independently of SA and *PR* gene activation, as SA-nonaccumulating NahG plants developed normal levels of ISR against *P. syringae* pv. *tomato* after colonization of the roots by WCS417r (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Similarly, the SA induction-deficient mutants *sid1-1* and *sid2-1* (Nawrath and Métraux, 1999) expressed WCS417r-mediated ISR (C.M.J. Pieterse, unpublished results), again demonstrating that WCS417r-mediated ISR is SA-independent. Using the JA response mutant *jar1-1*, the ET response mutant *etr1-1*, and the SAR regulatory mutant *npr1-1*, it was demonstrated that signal transduction leading to WCS417r-mediated ISR requires responsiveness to both JA and ET and, similar to pathogen-induced SAR, is dependent on NPR1 (Pieterse *et al.*, 1998). Like WCS417r, methyl jasmonate (MeJA) and the ET precursor 1-aminocyclopropane-1-carboxylate (ACC) were effective in inducing resistance against *P. syringae* pv. *tomato* in NahG plants. Moreover, MeJA-induced protection was blocked in *jar1-1*, *etr1-1*, and *npr1-1* plants, whereas ACC-induced protection was affected in *etr1-1* and *npr1-1* plants, but not in *jar1-1* plants. Hence, it was postulated that WCS417r-mediated ISR follows a novel signaling pathway in which components from the JA and ET response are successively engaged to trigger a defense reaction that, like SAR, is regulated by NPR1 (Pieterse *et al.*, 1998). Downstream of NPR1, *PR* genes are activated in the SAR pathway but not in the ISR pathway (Cao *et al.*, 1994; Pieterse *et al.*, 1998). Evidently, NPR1 differentially regulates ISR- and SAR-related gene expression, depending on the pathway that is activated upstream of it.

Production of JA and ET during ISR

Increased production of JA and ET is an early symptom of active defense in infected plants. Both signaling molecules coordinate the activation of a large set of defense responses, and when applied exogenously, can induce resistance themselves. In *Arabidopsis*, both JA and ET activate specific sets of defense-related genes and resistance against *P. syringae* pv. *tomato* (Van Wees *et al.*, 1999). Recently, we monitored the expression of a set of well-characterized JA- and/or ET-responsive genes in *Arabidopsis* plants expressing ISR. None of the genes tested were up-regulated in induced plants, neither locally in the roots, nor systemically in the leaves (Van Wees *et al.*, 1999). This suggests that WCS417r-mediated ISR in *Arabidopsis* was not associated with major changes in the levels of either JA or ET. Indeed, analysis of local and systemic levels of JA and ET revealed that WCS417r-mediated ISR is not associated with changes in the production of these signal molecules (Pieterse *et al.*, 2000). By using the *LOX2* co-suppressed transgenic line S-12, we confirmed that an increase in JA production is not required for the induction or expression of ISR. Transgenic S-12 plants, that are affected in the production of JA in response to wounding (Bell *et al.*, 1995), expressed normal levels of ISR (Pieterse *et al.*, 2000). Together, these results suggest that the JA and ET dependency of ISR is based on enhanced sensitivity to these hormones, rather than on an increase in their production.

Potentiation of JA-responsive genes in plants expressing ISR

If the JA and ET dependency of ISR is based on enhanced sensitivity to these signal molecules, ISR-expressing plants would be expected to react faster or more strongly to pathogen-induced JA or ET production. Therefore, the expression of the JA-responsive genes *AtVSP, PDF1.2, LOX2*, and *PAL1*, the ET-responsive genes *HEL* and *CHI-B*, and the SA-inducible genes *PR-1, PR-2*, and *PR-5* was analyzed after challenge of control, SAR- and ISR-expressing plants (Van Wees *et al.*, 1999). Infection with *P. syringae* pv. *tomato* induced the expression of all genes tested. In challenged, SAR-expressing plants the SA-inducible genes *PR-1, PR-2*, and *PR-5* showed a potentiated expression compared to challenged control plants. In challenged, ISR-expressing plants, only *AtVSP* displayed an enhanced level of expression in comparison to challenged control plants. The expression of the other JA-responsive genes was not potentiated, suggesting that ISR is associated with the potentiation of a specific set of JA-responsive genes.

ISR is associated with enhanced capacity for conversion of ACC to ET

In higher plants, ET is produced from methionine (Met) via *S*-adenosyl-L-methionine (SAM) and ACC (Met → SAM → ACC → ET; Kende and Zeevaart, 1997). The last two steps of this biosynthetic pathway are catalyzed by ACC synthase and ACC oxydase, respectively. Pathogen infections leading to chlorotic or necrotic symptoms cause an increase in ET production with ACC synthase and ACC oxidase activity being increased sequentially (De Laat and Van Loon, 1982). Under normal conditions the conversion of SAM to ACC is the rate-limiting step, however, during infections, ACC accumulates transiently, indicating that ACC oxidase activity restricts ET production. In *Arabidopsis*, ET production is not increased in systemic, ISR-expressing tissues compared to non-induced plants. However, after treatment with a saturating dose of 1 mM ACC, ISR-expressing plants showed a statistically significant higher level of ET emission than ACC-treated control plants (Pieterse *et al.*, 2000; S. Hase, unpublished results). The magnitude of the increase in ACC-converting capacity varied from 20 to 50% between experiments. Also, in the first 24 hours after inoculation with *P. syringae* pv. *tomato*, ISR-expressing plants showed a significant increase in ET emission (S. Hase, unpublished results). Evidently, the capacity to convert ACC to ET is increased in *Arabidopsis* plants expressing ISR, providing a greater potential for producing ET upon pathogen attack. As application of ACC has been shown to induce resistance against *P. syringae* pv. *tomato* in *Arabidopsis* (Pieterse *et al.*, 1998), a faster or greater production of ET in the initial phase of infection may contribute to enhanced resistance against this pathogen.

Spectrum of effectiveness of ISR and SAR

In *Arabidopsis*, SA, JA and ET are involved to different extents in basal resistance against specific pathogens. Basal resistance against the oomycetous pathogen *Peronospora parasitica* and to turnip crinkle virus (TCV) seems to be controlled predominantly by a SA-dependent pathway. Only SA-nonaccumulating *NahG* plants exhibited enhanced disease susceptibility to these pathogens (Delaney *et al.*, 1994; Kachroo *et al.*, 2000), whereas mutants affected in JA or ET signaling did not (Kachroo *et al.*, 2000; Thomma *et al.*, 1998). In contrast, basal resistance against the fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* was reduced only in JA- and ET-insensitive mutants, and not in NahG plants (Thomma *et al.*, 1998; 1999). Interestingly, basal resistance against the bacterial pathogens *P. syringae* pv. *tomato* and *Xanthomonas campestris* pv. *armoraciae* was found to be affected in both *NahG* plants and in JA- and ET-response mutants (Pieterse *et al.*, 1998; Ton *et al.*, 2001b), suggesting that basal resistance against these pathogens is controlled by a combined action of SA, JA and ET. To compare the effectiveness of SA-dependent SAR and JA/ET-dependent

ISR, we performed standard ISR and SAR bioassays using the different *Arabidopsis* pathogens that, in non-induced plants, are primarily resisted through either SA-dependent defenses, i.e. *P. parasitica* and TCV, JA/ET-dependent defenses, i.e. *A. brassicicola*, or a combination of SA-, JA-, and ET defenses, i.e. *P. syringae* pv. *tomato* and *X. campestris* pv. *armoraciae*. Induction of SAR and ISR was equally effective against *P. syringae* pv. *tomato* and *X. campestris* pv. *armoraciae*. In addition, activation of ISR resulted in a significant level of protection against *A. brassicicola*, whereas SAR was ineffective against this pathogen. Conversely, activation of SAR resulted in a high level of protection against *P. parasitica* and TCV, whereas ISR conferred only weak and no protection against *P. parasitica* and TCV, respectively. These results indicate that SAR is effective against pathogens that in non-induced plants are resisted through SA-dependent basal resistance responses, whereas ISR is effective against pathogens that in non-induced plants are resisted through JA/ET-dependent basal resistance responses (Ton *et al.*, 2001b; see also Ton *et al.* elsewhere in this issue).

ISR and SAR are additive

Cross-talk between defense signaling pathways has been demonstrated: JA and ET can act in concert in activating defense responses, whereas SA can suppress JA-dependent responses (Pieterse *et al.*, 2001b). Together with the fact that ISR and SAR share the regulatory factor NPR1, the question was raised as to what extent the JA-dependent ISR pathway and the SA-dependent SAR pathway interact. To investigate possible interactions between the ISR and the SAR pathway, we induced ISR and SAR against *P. syringae* pv. *tomato* simultaneously. Interestingly, simultaneous activation of both pathways resulted in an additive effect on the level of induced protection (Van Wees *et al.*, 2000). In *Arabidopsis* genotypes that are blocked in either SAR or ISR, this additive effect was not evident. Moreover, expression of the SAR marker gene *PR-1* was not altered in plants expressing both ISR and SAR compared to plants expressing SAR alone, indicating that the SAR and the ISR pathway are compatible and that there is no significant cross-talk between these signaling pathways.

Search for rhizobacteria-mediated ISR-related genes

The state of pathogen-induced SAR is characterized by the concomitant activation of a large set of genes (Maleck *et al.*, 2000). Of many defense-related genes tested in *Arabidopsis* (e.g. the SA-inducible genes *PR-1*, *PR-2*, and *PR-5*, and the ET- and/or JA-inducible genes *HEL*, *CHI-B*, *PDF1.2*, *AtVSP*, *LOX1*, *LOX2*, and *PAL1*), none were found to be up-regulated in plants expressing ISR (Van Wees *et al.*, 1999). Thus, in contrast to SAR, the onset of ISR is not associated with major changes in gene expression. Nevertheless, ISR-expressing plants are clearly more resistant to different types of pathogens. Therefore, plants must possess as yet undiscovered defense-related gene products that contribute to broad-spectrum resistance.

In another approach to search for ISR-related genes, a large collection of *Arabidopsis* lines containing enhancer-trap *Ds* transposons and the β-glucuronidase (GUS) reporter gene were screened. One enhancer-trap line showed local GUS activity in the roots upon colonization by WCS417r (see also K.M. Léon-Kloosterziel *et al.* elsewhere in this issue). Interestingly, the roots of this line showed a similar expression pattern after treatment of the roots with the ET precursor ACC, indicating that this line contains a transposon insertion in the vicinity of an ET-inducible gene that is up-regulated in the roots upon colonization by WCS417r. Characterization of the gene revealed that it encodes a thaumatin-like gene. Thaumatins have repeatedly been implicated in plant defense. Currently, we are investigating the role of this gene in ISR.

Identification of a novel locus (ISR1) controlling rhizobacteria-mediated ISR

In a genetic approach to identify ISR-related genes, we screened 10 accessions of *Arabidopsis* for their potential to express ISR and SAR against *P. syringae* pv. *tomato* (Ton *et al.*, 1999). All accessions tested developed SAR. However, of the 10 accessions tested, RLD and Wassilewskija did not develop ISR after treatment of the roots with WCS417r. The WCS417r-nonresponsive phenotype was associated with a relatively high susceptibility to *P. syringae* pv. *tomato*. Genetic analysis of the F_1, F_2, and F_3 progeny of a cross between the WCS417r-responsive accession Columbia and the WCS417r-nonresponsive accession RLD, revealed that both the potential to express ISR and the relatively high level of basal resistance against *P. syringae* pv. *tomato* are monogenic, dominant traits that are genetically linked. The corresponding locus, designated *ISR1*, was mapped between CAPS markers *B4* and *GL1* on chromosome III. Neither responsiveness to WCS417r, nor the relatively high level of basal resistance was complemented in the F_1 progeny of crosses between RLD and Wassilewskija, indicating that both accessions are affected in the same locus.

Interestingly, mutants *jar1-1* and *etr1-1*, that are affected in their response to JA and ET, respectively, showed the same phenotype as accessions RLD and Wassilewskija in that they were both unable to express WCS417r-mediated ISR and showed enhanced susceptibility to *P. syringae* pv. *tomato* infection (Pieterse *et al.*, 1998). Analysis of ET-responsiveness of RLD and Wassilewskija revealed that both accessions showed a reduced sensitivity to ET, that co-segregated with the recessive alleles of the *ISR1* locus (Ton *et al.*, 2001a). Therefore, it is proposed that the *Arabidopsis ISR1* locus encodes a novel component of the ET-response pathway that plays an important roie in disease-resistance signaling.

Concluding remarks

Recent advances in research on plant defense signaling pathways have shown that plants are capable of differentially activating distinct defense pathways, depending on the type of invader encountered (Pieterse and Van Loon, 1999; Pieterse *et al.*, 2001b). Salicylic acid is an important signaling molecule in both locally and systemically induced resistance responses. However, research on rhizobacteria-mediated ISR signaling in *Arabidopsis* demonstrated that JA and ET play key the roles. During the past five years, research on rhizobacteria-mediated ISR has increased our knowledge of the molecular mechanisms involved in this form of induced disease resistance. An important conclusion is that different rhizobacteria utilize different mechanisms for triggering systemic resistance: some rhizobacteria trigger a SA-dependent pathway, others a JA/ET-dependent pathway (Pieterse *et al.*, 2001a). In this respect, it is interesting to note that simultaneous activation of the SA-dependent SAR pathway and the JA/ET-dependent ISR pathway resulted in an additive effect on the level of induced resistance attained (Van Wees *et al.*, 2000). Therefore, combining rhizobacterial strains that trigger different signaling pathways in the plant provides an attractive possibility for the improvement of disease control (see also Van Loon *et al.* elsewhere in this issue).

In contrast to SAR, rhizobacteria-mediated ISR in *Arabidopsis* is not associated with major changes in gene expression. Currently, research on the molecular mechanisms underlying ISR is hampered by the lack of reliable molecular markers. Therefore, future research will be focussed on identifying such marker genes using techniques such as screening of DNA microarrays, screening of enhancer/gene-trap lines, and map-based cloning approaches. Furthermore, the mechanisms involved in potentiation of JA-responsive gene expression and the increased ACC-converting capacity in ISR-expressing plants need to be investigated. Both latter findings are examples of priming that may lead to a faster and/or enhanced activation of JA- and ET-dependent defense reactions upon attack by a challenging

pathogen. If priming of defense responses plays an important role in ISR, then this could explain the absence of major changes in defense-related gene expressing prior to challenge. Investigations of these phenomena will be most challenging and will certainly provide more insight in the molecular mechanisms of induced disease resistance.

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