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Identification of genes involved in rhizobacteria-mediated induced systemic resistance in *Arabidopsis*

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Abstract: Different forms of biologically induced disease resistance have been identified in plants. Following attack by a necrotizing pathogen systemic acquired resistance (SAR) is induced, leading to a broad-spectrum disease resistance that is associated with an increase in salicylic acid (SA) levels and the accumulation of pathogenesis-related (PR) proteins. Selected strains of non-pathogenic, root-colonizing fluorescent *Pseudomonas* spp. can induce systemic resistance as well, without provoking any symptoms themselves. This rhizobacteria-mediated induced systemic resistance (ISR) is phenotypically similar to pathogen-induced SAR in that it is effective against various pathogens. In *Arabidopsis thaliana*, the ISR signaling pathway triggered by *Pseudomonas fluorescens* WCS417r requires responsiveness to both jasmonate (JA) and ethylene (ET) and is independent of SA and the accumulation of PR proteins. The state of pathogen-induced SAR is characterised by the concomitant activation of a set of *PR* genes. Of many defence-related genes tested in *Arabidopsis*, none were up-regulated prior to challenge in plants expressing WCS417r-mediated ISR. In an attempt to isolate ISR-related genes, we screened a large collection of *Arabidopsis* lines containing enhancer-trap *Ds* transposons and the ß-glucuronidase (GUS) reporter gene with minimal promoter. One enhancer-trap line showed local GUS activity in the roots upon colonization with WCS417r. This local GUS expression was not observed after treatment of the roots with *Escherichia coli*, indicating that the induction was *Pseudomonas* specific. Interestingly, a similar expression pattern was observed 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 upon colonization with WCS417r. There are several candidate genes in the vicinity of the enhancer-trap *Ds* transposon, one of which encodes a thaumatin-like protein. Gene expression analyses confirmed that this thaumatin-like gene, designated *THL1*, is up-regulated in response to treatment of the roots with WCS417r or ACC. Analysis of the role of *THL1* in ISR might provide more insight into the molecular mechanisms involved in rhizobacteria-mediated ISR.

Key words: defense-related gene, ethylene, ISR, *Pseudomonas*, SAR, thaumatin

Introduction

Plants possess multiple inducible defense mechanisms to defend themselves against pathogen attack. A classic example of such an induced resistance is activated after primary infection with a necrotizing pathogen, rendering distant, uninfected plant parts more resistant towards a broad spectrum of virulent pathogens (Kuc, 1982). This form of induced resistance is often referred to as systemic acquired resistance (SAR; Ross, 1961), and has been demonstrated in many plant-pathogen interactions. The state of SAR is characterized by an early increase in salicylic acid (SA) levels and the activation of a specific set of genes encoding pathogenesis-related (PR) proteins (Ryals *et al.*, 1996; Sticher *et al.*, 1998; Van Loon, 1997). Another form of induced disease resistance is triggered by selected strains of non-pathogenic rhizobacteria. Phenotypically, ISR resembles pathogen-induced SAR in that it is effective against a broad spectrum of plant pathogens (Van Loon *et al.*, 1998). Previously, we developed an *Arabidopsis*-

based model system to study the molecular basis underlying rhizobacteria-mediated ISR (for review see Pieterse *et al.*, 2001 and Pieterse *et al.* elsewhere in this issue). In *Arabidopsis*, the signaling pathways of ISR and SAR clearly diverge. While SAR requires SA, ISR is dependent on an intact response to the plant hormones jasmonic acid (JA) and ethylene (ET) (Pieterse *et al.*, 1998). Of the many defence-related genes tested in *Arabidopsis* none are up-regulated in plants expressing ISR, prior to pathogen attack (Pieterse *et al.*, 1996; 1998; Van Wees *et al.*, 1999). Moreover, standard differential screening of a cDNA library of *P. fluorescens* WCS417r induced plants did not yield consistent significant differences. 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 genes whose products contribute to broad-spectrum disease resistance. In an attempt to identify ISR-related genes we screened a collection of *Arabidopsis* lines containing randomly inserted enhancer-trap *Ds* transposons with the ß-glucuronidase (*GUS*) reporter gene with a minimal promoter.

Material and methods

Plant and bacterial material

Arabidopsis Ds gene-trap (WGT) and *Ds* enhancer-trap (WET) lines were kindly provided by dr. Vroemen (Wageningen University, The Netherlands). This collection was generated in the background of *Arabidopsis* accession Landsberg *erecta* (*Ler*) (Vroemen *et al.*, 1998). Seeds were sown on MS-agar plates and after 11-12 days transferred to soil containing *P. fluorescens* WCS417r ($5x10^7$ cfu/g soil) as described (Pieterse *et al.*, 1996). Four and 11 days after transfer, seedlings were analyzed for GUS expression as described by Vroemen *et al.* (1998). The assessment of ISR and rhizosphere colonization were performed as described (Pieterse *et al.*, 1996).

Histochemical localization of GUS expression

Plants were immersed in GUS staining solution. After o/n incubation, the plants were destained and observed under a stereomicroscope. Seeds were sown on MS-agar plates. After 12 days, 1 mM ACC was added onto the plates and GUS expression determined 6 days later. For the determination of GUS expression after root colonization on artificial media, seeds were sown on half-strength Hoagland nutrient-agar plates. After 14 days, a bacterial suspension (10^7 cfu/ml) of WCS417r or *E. coli* strain S17 was applied at the base of the hypocotyl and GUS expression determined after 4 days.

Southern blot analysis, RNA blot analysis, RT-PCR and TAIL-PCR

Genomic DNA was extracted from 3-week-old rosettes. The DNA was digested, separated, and blotted onto a membrane. The *GUS* coding sequence was labeled and used as probe. For RNA gel blot analysis and RT-PCR analysis, 12-day old seedlings grown on MS-agar plates treated with 100 µM ACC. Genomic sequences of the *Hsp90*, the *TLP1*, the *KHCP*, and the β-tubulin gene were used as probes. For RT-PCR, polyA$^+$ RNA was extracted from the ACC-treated and control roots. Equal amounts of cDNA were prepared and used in a PCR with the gene specific primers. To amplify genomic sequences flanking the *DsE* insertion in WET121, TAIL-PCR was performed essentially as described by Liu *et al.* (1995).

Results and discussion

A large collection of gene trap and enhancer trap lines (WGT and WET lines, Wageningen) was used in a screen to identify genes associated with the onset of ISR. The starter lines of

this collection were tested for their ability to express ISR after treatment of the roots with *P. fluorescens* WCS417r, using *Pseudomonas syringae* pv. *tomato* as the challenging pathogen. Wild-type L*er* plants showed a 50-80% reduction of disease symptoms in response to WCS417r-treatment, which is in agreement with previous results (Van Wees *et al.*, 1997; Ton *et al.*, 1999). WCS417r-treated starter-lines showed a similar reduction of disease symptoms, indicating that they are capable of expressing ISR.

Plants of 200 gene-trap and 400 enhancer-trap lines were screened for GUS expression after colonization of the roots with WCS417r. Since the exact time point at which ISR is established is not known, the GUS expression assay was performed at different time-points after transferring the plants to the soil (e.g. 4 and 11 days). From all lines screened, one enhancer trap line (WET121) showed GUS activity at 4 days after root colonization. The blue staining was present at dispersed spots along the roots, but not in the rosette or the hypocotyl. Control WET121 plants grown in soil without bacteria did not show any GUS activity. When WET121 plants were grown on agar-nutrient medium plates, the roots showed similar expression patterns after root colonization with WCS417r, but no GUS expression was seen after inoculation with *E. coli*. In *E. coli*-treated plants some blue staining was visible at the surface of root hairs caused by the bacterial endogenous GUS-activity, but this was clearly different from the dispersed spots visible after WCS417r colonization. These results indicate that in WET121 plants the *Ds* transposon is inserted in the vicinity of a root specific gene, which is induced in response to colonization by ISR-inducing WCS417r bacteria.

JA and ET are important signal molecules in the ISR signaling pathway, whereas SA plays a dominant role in the SAR pathway. The involvement of these signal molecules in the induction of the GUS activity in WET121 was assayed. When plants were grown on media containing MeJA or SA, no GUS expression was observed, but when WET121 was grown on media containing the ethylene precursor ACC, GUS activity was observed in large parts of the root. Microscopic evaluation of GUS staining in WCS417r- and ACC-treated plants revealed that the *GUS* gene expression was localized in the vascular tissue of the roots.

In enhancer trap lines, GUS expression can be activated when the *DsE* element, containing the *GUS* gene, is inserted within or outside the coding region of chromosomal genes. Southern blot analysis showed that the *DsE* element in WET121 was present in a single copy. The flanking DNA was amplified using TAIL-PCR and sequenced. Alignment of the two flanking sequences with the *Arabidopsis* genome sequence showed that the *DsE* element was inserted in chromosome 4. There are three genes in the vicinity of the *DsE* insertion: a gene encoding heat shock protein Hsp90, a gene encoding a thaumatin-like protein, designated *THL1*, and a gene encoding a kinase heavy chain protein (KHCP). RNA blot and RT-PCR analyse revealed that only the *TLP1* gene was coordinately expressed with the *GUS* gene. This indicates that in WET121 the *TLP1* is up-regulated in response to WCS417r and ACC treatment.

The predicted *THL1* gene product is a 288 amino acid protein with a putative signal peptide and several predicted phosphorylation sites. The TLP1 protein shows significant homology to PR-5 of *Arabidopsis* and osmotin of tomato. Conserved among these proteins are 16 cysteine residues that form 8 pairs of disulfide bonds to stabilize the protein structure (Kitajama and Soto, 1999). Currently, we are investigating the role of TLP1 in ISR, which might provide more insight in the molecular mechanisms of rhizobacteria-mediated ISR.

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