

Colonization of the *Arabidopsis* rhizosphere by fluorescent *Pseudomonas* spp. activates a root-specific, ethylene-responsive PR-5 gene in the vascular bundle

Karen M. Léon-Kloosterziel^{1,3,†}, Bas W.M. Verhagen^{1,4,†}, Joost J.B. Keurentjes^{1,5}, Johan A. VanPelt¹, Martijn Rep², L.C. VanLoon¹ and Corné M.J. Pieterse^{1,*}

¹Graduate School Experimental Plant Sciences, Section Phytopathology, Faculty of Biology, Utrecht University, P.O. Box 800.84, 3508 TB Utrecht, the Netherlands (*author for correspondence; e-mail C.M.J.Pieterse@bio.uu.nl, <http://www.bio.uu.nl/~fytopath>); ²Plant Pathology, Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam, P.O. Box 94062, 1090 GB Amsterdam, the Netherlands; ³present address: Department of Cell Biology and Immunology, Wageningen University, P.O. Box 338, 6700 AH Wageningen, the Netherlands; ⁴present address: Laboratoire d'Eco-Toxicologie, UFR Science Exactes et Naturelles, Université de Reims, Moulin de la Housse, BP1039 – 51687, Reims, France; ⁵present address: Laboratory of Genetics, Wageningen University, Arboretumlaan 4, 6703 BD, Wageningen, the Netherlands; [†]The authors contributed equally to this work

Received 25 October 2004; accepted in revised form 28 February 2005

Key words: defense-related gene expression, enhancer trap, ethylene, ISR, *Pseudomonas fluorescens*, thaumatin-like protein

Abstract

Plants of which the roots are colonized by selected strains of non-pathogenic, fluorescent *Pseudomonas* spp. develop an enhanced defensive capacity against a broad spectrum of foliar pathogens. In *Arabidopsis thaliana*, this rhizobacteria-induced systemic resistance (ISR) functions independently of salicylic acid but requires responsiveness to jasmonic acid and ethylene. In contrast to pathogen-induced systemic acquired resistance (SAR), ISR is not associated with systemic changes in the expression of genes encoding pathogenesis-related (PR) proteins. To identify genes that are specifically expressed in response to colonization of the roots by ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria, we screened a collection of *Arabidopsis* enhancer trap and gene trap lines containing a transposable element of the *Ac/Ds* system and the *GUS* reporter gene. We identified an enhancer trap line (WET121) that specifically showed *GUS* activity in the root vascular bundle upon colonization of the roots by WCS417r. Fluorescent *Pseudomonas* spp. strains *P. fluorescens* WCS374r and *P. putida* WCS358r triggered a similar expression pattern, whereas ISR-non-inducing *Escherichia coli* bacteria did not. Exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) mimicked the rhizobacteria-induced *GUS* expression pattern in the root vascular bundle, whereas methyl jasmonic acid and salicylic acid did not, indicating that the *Ds* element in WET121 is inserted in the vicinity of an ethylene-responsive gene. Analysis of the expression of the genes in the close vicinity of the *Ds* element revealed *AtTLPI* as the gene responsible for the in *cis* activation of the *GUS* reporter gene in the root vascular bundle. *AtTLPI* encodes a thaumatin-like protein that belongs to the PR-5 family of PR proteins, some of which possess antimicrobial properties. *AtTLPI* knockout mutant plants showed normal levels of WCS417r-mediated ISR against the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000, suggesting that expression of *AtTLPI* in the roots is not required for systemic expression of ISR in the leaves. Together, these results indicate that induction of *AtTLPI* is a local response of *Arabidopsis* roots to colonization by non-pathogenic fluorescent *Pseudomonas* spp. and is unlikely to play a role in systemic resistance.

Introduction

Selected strains of non-pathogenic, root-colonizing bacteria can stimulate plant growth and are, therefore, referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1980). Plant growth promotion results mainly from suppression of soil-borne pathogens and other deleterious micro-organisms, but also direct effects on plant growth have been reported (Schippers *et al.*, 1987). Fluorescent *Pseudomonas* spp. are among the most effective suppressors of disease and have been shown to be responsible for the reduction of soil-borne diseases in naturally disease-suppressive soils (Raaijmakers and Weller, 1998). Disease suppressive activity can be the result of competition for nutrients, siderophore-mediated competition for iron, antibiosis, or the production of lytic enzymes (Van Loon and Bakker, 2003).

Apart from direct antagonistic effects on soil-borne pathogens, some rhizobacterial strains are able to reduce disease in above-ground plant parts through a plant-mediated mechanism that is commonly referred to as induced systemic resistance (ISR) (Van Loon *et al.*, 1998). Rhizobacteria-mediated ISR has been demonstrated in many plant species, and is effective against a broad spectrum of plant pathogens, including oomycetes, fungi, bacteria and viruses (Van Loon *et al.*, 1998). Phenotypically, rhizobacteria-mediated ISR resembles classic pathogen-induced resistance, in which non-infected parts of infected plants become more resistant to further infection. This latter form of induced resistance is known as systemic acquired resistance (SAR) (Ross, 1961; Sticher *et al.*, 1997).

Pseudomonas fluorescens strain WCS417r has been shown to trigger ISR in several plant species, e.g. carnation (Van Peer *et al.*, 1991), radish (Leeman *et al.*, 1995), tomato (Duijff *et al.*, 1998), bean (Bigirimana and Höfte, 2002) and *Arabidopsis* (Pieterse *et al.*, 1996; Pieterse *et al.*, 2002). Colonization of *Arabidopsis* roots by WCS417r protects the plant systemically against different types of pathogens, including the bacterial leaf pathogens *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Xanthomonas campestris* pv. *armoraciae*, the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani*, the fungal leaf pathogen *Alternaria brassicicola*, and the oomycete leaf pathogen *Peronospora parasitica* (Pieterse *et al.*,

1996; Van Wees *et al.*, 1997; Ton *et al.*, 2002b). The spectrum of effectiveness of WCS417r-mediated ISR and pathogen-induced SAR overlaps, but is also partly divergent. For instance, ISR is effective against *A. brassicicola*, whereas SAR is not. Conversely, SAR is effective against turnip crinkle virus (TCV), whereas ISR is not (Ton *et al.*, 2002b). In some cases, the level of induced protection can be enhanced further when both ISR and SAR are activated simultaneously (Van Wees *et al.*, 2000), indicating that the effects of both types of induced resistance are additive.

It has been established that the signal transduction pathways of rhizobacteria-mediated ISR and pathogen-induced SAR are distinct. The state of SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA), and the concomitant activation of a set of so-called SAR genes (Sticher *et al.*, 1997). Many SAR genes encode pathogenesis-related (PR) proteins (PRs) (Van Loon and Van Strien, 1999), some of which have been shown to possess anti-fungal activity and are thought to contribute to the induced resistant state. *Arabidopsis* mutants affected in SA production, and transgenic NahG plants that express the bacterial salicylate hydroxylase (*nahG*) gene, are incapable of developing SAR and do not show SAR gene activation upon pathogen infection, indicating that SA is a necessary intermediate in the SAR signaling pathway (Gaffney *et al.*, 1993; Wildermuth *et al.*, 2001; Nawrath *et al.*, 2002). Transduction of the SA signal requires the function of the regulatory protein NPR1 (Dong, 2004; Pieterse and Van Loon, 2004). Interaction of NPR1 with b-ZIP TGA transcription factors is required for activation of the SA-regulated PR-genes, suggesting that NPR1 acts by altering the activity of transcription factors (Dong, 2004).

Some rhizobacteria trigger the SA-dependent SAR pathway by producing SA on the root (De Meyer *et al.*, 1999; Maurhofer *et al.*, 1998). However, WCS417r induces normal levels of protection against *Pst* strain DC3000 in *Arabidopsis* genotypes that are impaired in SA accumulation (i.e. NahG, *eds5-1*, *sid2-1*) (Pieterse *et al.*, 1996; Pieterse *et al.*, 2002; Ton *et al.*, 2002a). Moreover, WCS417r-mediated ISR in wild-type *Arabidopsis* plants is not accompanied by activation of the SAR marker genes *PR-1*, *PR-2*, and *PR-5* (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997).

Thus, this type of induced resistance functions independently of SA. 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 WCS417r-mediated ISR, but that like SAR, this induced resistance response depends on NPR1 (Knoester *et al.*, 1999; Pieterse *et al.*, 1998). However, downstream of NPR1, the ISR and the SAR signaling pathways diverge, because unlike SAR, ISR is not accompanied by the concomitant activation of *PR* genes (Pieterse *et al.*, 1996; Van Wees *et al.*, 1999; Van Wees *et al.*, 1997). Apart from WCS417r, other fluorescent *Pseudomonas* spp. strains have also been shown to induce SA-independent ISR in *Arabidopsis* (Van Wees *et al.*, 1997; Iavicoli *et al.*, 2003; Ryu *et al.*, 2003), tobacco (Press *et al.*, 1997; Zhang *et al.*, 2002) and tomato (Yan *et al.*, 2002), indicating that the ability to trigger an SA-independent pathway controlling systemic resistance is not uncommon among ISR-inducing rhizobacteria.

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

To identify ISR-related genes, we recently surveyed the transcriptional response of over 8,000 *Arabidopsis* genes during rhizobacteria-mediated ISR (Verhagen *et al.*, 2004). However,

systemically in the leaves, none of the ~8000 genes tested showed a consistent change in expression in response to effective colonization of the roots by WCS417r, indicating that the onset of ISR in the leaves is not associated with detectable changes in gene expression. After challenge inoculation of WCS417r-induced plants with the bacterial leaf pathogen *Pst* DC3000, 81 genes showed an augmented expression pattern in ISR-expressing leaves compared to inoculated control leaves, suggesting that ISR-expressing plants are primed to respond faster and/or more strongly upon pathogen attack. The majority of the identified potentiated genes was predicted to be regulated by JA and/or ET signaling. Priming is a process that provides the plant with an enhanced capacity for rapid and effective activation of cellular defense responses that are induced only after contact with a pathogen (Conrath *et al.*, 2002). It allows the plant to react more effectively to the invader encountered, which might explain the broad-spectrum action of induced resistance.

In a complementary approach to identify novel defense-related genes that are potentially involved in rhizobacteria-mediated ISR, we screened a collection of gene trap and enhancer trap lines with transposable elements of the *Ac/Ds* system carrying the β -glucuronidase (*GUS*) reporter gene (Sundaresan *et al.*, 1995; Vroemen *et al.*, 1998), for genes that are specifically expressed in response to colonization of the roots by ISR-inducing WCS417r bacteria. Here, we identified a thaumatin-like gene (*AtTLP1*) that is specifically expressed in the root vascular bundle after colonization by fluorescent *Pseudomonas* spp., and after treatment with the ET precursor 1-aminocyclopropane-1-carboxylate (ACC). The putative thaumatin-like protein shows high homology to members of the PR-5 group of PR proteins.

Materials and methods

Bacterial and fungal cultures

Non-pathogenic, rifampicin-resistant *Pseudomonas fluorescens* strain WCS417r was used for induction of ISR and for screening the enhancer trap and gene trap collection. WCS417r was grown for 24 h at 28 °C on Kings medium B agar plates (King *et al.*, 1954) containing the appropriate

antibiotics as described previously (Pieterse *et al.*, 1996). Subsequently, bacteria were collected and resuspended in 10 mM MgSO₄ to a density of 10⁹ cfu ml⁻¹ (OD₆₆₀ = 1.0) before being mixed through the soil. Growth of *Pseudomonas fluorescens* strain WCS374r, *Pseudomonas putida* strain WCS358r, and *Escherichia coli* strain S17 for analysis of *GUS* expression in WET121 was performed in a similar manner.

An avirulent strain of *P. syringae* pv. *tomato* DC3000 carrying the avirulence gene *avrRpt2* (*Pst* DC3000(*avrRpt2*)) (Kunkel *et al.*, 1993) was used for induction of SAR. *Pst* DC3000(*avrRpt2*) bacteria were grown overnight at 28 °C in liquid KB supplemented with 25 mg ml⁻¹ kanamycin to select for the plasmid. After centrifugation, the bacterial cells were resuspended in 10 mM MgSO₄ to a final density of 10⁷ cfu ml⁻¹. Virulent *Pst* DC3000 (Whalen *et al.*, 1991), used for challenge inoculations, was cultivated in a similar manner in liquid KB without kanamycin. After centrifugation, the bacterial cells were resuspended in 10 mM MgSO₄, 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, the Netherlands) to a final density of 2.5 × 10⁷ cfu ml⁻¹.

Plant growth conditions

For induction of ISR or isolation of RNA or DNA from leaves, seeds of wild-type *Arabidopsis thaliana* accessions Landsberg *erecta* (*Ler-0*), enhancer trap line WET121, starter lines *Ac2* and *DsE3* (Vroemen *et al.*, 1998), and *AtTLP1* knock-out mutant line SGT5141 (Parinov *et al.*, 1999) were sown in quartz sand. WET121, SGT5141, *Ac2* and *DsE3* lines were in the *Ler-0* background. Two-week-old seedlings were transplanted into 60-ml pots containing a sand/potting soil mixture (5:12, v/v) that had been autoclaved twice for 20 min with a 24-h interval. Prior to transfer of the *Arabidopsis* seedlings, a suspension of ISR-inducing WCS417r bacteria (10⁹ cfu ml⁻¹) was mixed thoroughly through the soil to a final density of 5 × 10⁷ cfu g⁻¹ as described previously (Pieterse *et al.*, 1996). Control soil was supplemented with an equal volume of 10 mM MgSO₄.

For isolation of RNA from roots, 2-week-old seedlings were laid out horizontally on rock-wool cubes (Rock-wool/Grodan B.V., Roermond, the Netherlands) as described previously (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997). Subsequently,

the root systems were covered with 1 ml of a 1:1 (w/v) mixture of talcum powder and either a suspension of ISR-inducing WCS417r in 10 mM MgSO₄ (final density 5 × 10⁸ cfu g⁻¹), or a solution of 10 mM MgSO₄ as a control. Plants were cultivated in a growth chamber with a 9-h day (200 μE m⁻² s⁻¹ at 24 °C) and a 15-h night (20 °C) cycle at 70% relative humidity.

Plants received a modified half-strength Hoagland nutrient solution (2 mM KNO₃, 5 mM Ca(NO₃)₂, 1 mM KH₂PO₄, 1 mM MgSO₄, and trace elements, pH 7 (Hoagland and Arnon, 1938)) once a week as described previously (Pieterse *et al.*, 1996).

Bioassays

Induction of ISR was performed by mixing ISR-inducing WCS417r bacteria through the soil as described above. Induction of SAR was performed 3 days before challenge inoculation by pressure infiltrating three lower leaves with a suspension of avirulent *Pst* DC3000(*avrRpt2*) bacteria at 10⁷ cfu ml⁻¹ as described previously (Pieterse *et al.*, 1996). For assaying induced resistance, plants were challenged when 5 weeks old by dipping the leaves for 2 s in a suspension of virulent *Pst* DC3000 bacteria at 2.5 × 10⁷ cfu.ml⁻¹ in 10 mM MgSO₄, 0.015% (v/v) Silwet L-77. 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. From the number of diseased and non-diseased leaves, the disease index was calculated for each plant (*n* = 20). Colonization levels of the rhizosphere of the tested plants by rifampicin-resistant WCS417r bacteria were determined at the end of ISR bioassays as described (Pieterse *et al.*, 1996). In all cases, the number of colony-forming units per gram of root fresh weight ranged between 10⁶ and 10⁷ (data not shown), which is well above the threshold level required for induction of ISR (Van Loon *et al.*, 1998).

Gene trap and enhancer trap screening

For the *Arabidopsis* gene trap and enhancer trap screening, a collection of 200 Wageningen Gene

Trap (WGT) lines and 400 Wageningen Enhancer Trap (WET) lines (in *Ler-0* background) was used that was kindly provided by Dr C.W. Vroemen (Wageningen University, The Netherlands). Construction of the gene trap and enhancer trap lines (Vroemen *et al.*, 1998) was based on transposable elements of the *Ac/Ds* system carrying the *GUS* reporter gene (Sundaresan *et al.*, 1995). For the selection procedure, seeds were surface sterilized with 2% (w/v) sodium hypochlorite in 50% (v/v) ethanol and sown on 0.6% (w/v) agar plates containing 0.46% (w/v) Murashige and Skoog (MS) salts (Duchefa BV, Haarlem, the Netherlands), 1.0% (w/v) sucrose and 50 mg l⁻¹ kanamycin to select for plants with the gene/enhancer trap construct among the heterozygous lines. The plates were incubated in a growth cabinet with an 9-h day (24 °C) and 15-h night (20 °C) cycle. After 11–12 days, the seedlings were transferred to soil with or without ISR-inducing WCS417r bacteria as described above. Four and eleven days after transfer to soil, whole seedlings were removed from the soil, rinsed with tap water to remove excess soil particles from the roots, and analyzed for *GUS* expression.

For histochemical localization of *GUS* activity, whole plants were immersed in *GUS* staining solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) in 100 mM NaPi buffer pH 7.0, 10 mM EDTA, 0.1% Triton X-100 as described (Spoel *et al.*, 2003). After overnight incubation at 37 °C, the plants were destained by repeated washes in 70% ethanol and inspected under a stereomicroscope. For differential interference contrast microscopy, the *GUS* staining solution was complemented with 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide, and the plants were incubated at 37 °C for 3 days. After destaining, the plants were cleared by incubation in clearing solution (7 g chloralhydrate, 1 ml glycerol, 2 ml water) for 2–16 h.

GUS expression in WET121

Homozygous WET121 seeds were surface sterilized and sown on MS-agar plates supplemented with 0.1 mM SA, 0.01 mM MeJA, or 0.1 mM ACC as described (Spoel *et al.*, 2003; Ton *et al.*, 2002a). Plants were grown for 14 days prior to determination of *GUS* activity. Because ACC

inhibits growth, alternatively, seeds were sown on MS-agar plates as described above and after 12 days 2 ml of a solution containing 1 mM ACC was added. *GUS* expression was determined 6 days after ACC application. MeJA was purchased from Serva Brunschwig (Amsterdam, the Netherlands), ACC from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands) and SA from Mallinckrodt Baker BV (Deventer, the Netherlands).

To determine the effect of bacterial treatments, seeds were sown on 0.7%-agar plates containing half-strength Hoagland nutrients and 10 μM Sequestreen (Ciba Geigy, Basel, Switzerland). The agar plates were incubated vertically to allow development of the root system along the surface of the agar plates. After 14 days, 1 μl of a bacterial suspension containing 10⁷ cfu ml⁻¹ of WCS417r was applied at the base of the hypocotyl, resulting in colonization of the whole root system within 24 h. Four days later, *GUS* expression was determined as described above. Analysis of *GUS* expression in response to treatment of the roots with *P. fluorescens* WCS374r, *P. putida* WCS358r, or *E. coli* strain S17 was performed in a similar manner.

Analysis of flanking sequences of the Ds insertion in WET121

Isolation of genomic DNA and Southern blot analysis were performed as described previously (Vroemen *et al.*, 1998). To amplify genomic sequences flanking the *Ds* insertion in WET121, TAIL-PCR was performed essentially as described (Liu *et al.*, 1995; Vroemen *et al.*, 1998). In brief, two arbitrary degenerate (AD) primers, and three specific nested *Ds* primers were used to amplify the *Ds* flanking sequences in a primary, a secondary and a tertiary round of TAIL-PCR. In the consecutive rounds of TAIL-PCR, the positions of the *Ds* primers were chosen closer to the 5'-end of the *Ds* element. This resulted in consecutively smaller PCR products, indicative of a successful amplification of flanking sequences. Primers used were the nested specific primers located on the 5'-end of the *Ds* element: Ds5-1, 5'-CCG TTT ACC GTT TTG TAT ATC CCG-3'; Ds5-2, 5'-CGT TCC GTT TTC GTT TTT TAC C-3'; Ds5-3, 5'-GGT CGG TAC GGA ATT CTC CC-3'; and the arbitrary degenerate (AD) primers AD-2, 5'-NGT

CGA (C/G)(A/T)G ANA (A/T)GA A-3', AD-3, 5'-(A/T)GT GNA G(A/T)A NCA NAG A-3'. The products from the tertiary PCR reaction were separated on an agarose gel, isolated from the gel, cloned into pGEM-T-easy vector (Promega), and sequenced using universal T7 and SP6 sequencing primers.

Alignment software and phylogenetic tree construction

Alignment of amino acid sequences was performed using MegAlign software (DNASTar). Phylogenetic tree construction was done with MacVector (Oxford Molecular Group) as described previously (Rep *et al.*, 2002).

Northern blot analysis and RT-PCR

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 g plant tissue). The homogenates were extracted with phenol/chloroform/isoamylalcohol (25:24:1; v/v/v) and the RNA was precipitated using LiCl, as described (Sambrook *et al.*, 1989). Analysis of gene expression in the roots was performed by RT-PCR as described earlier (Pieterse *et al.*, 1998). Analysis of gene expression in the leaves was performed by northern blot analysis. To this end, 15 µg of RNA was denatured using glyoxal and DMSO as described (Sambrook *et al.*, 1989). Subsequently, samples were electrophoretically separated on 1.5% agarose gels and blotted onto Hybond-N⁺ membranes (Amersham, s-Hertogenbosch, the Netherlands) by capillary transfer. Northern blots were hybridized with gene-specific probes as described previously (Pieterse *et al.*, 1998). Templates for the preparation of gene-specific probes were prepared by PCR with the primers described below. After hybridization with α-³²P-dCTP-labelled probes, blots were subjected to autoradiography and signals quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, the Netherlands) with Quantity One software (BioRad, Veenendaal, the Netherlands).

For detection of the *AtTLP1* transcript, the following gene-specific primers were designed based on the annotated sequences corresponding to AGI number At4g24180: AtTLP1-FOR

5'-AGC ATT GCA TTT GGT AGG AAG TGA-3', AtTLP1-REV 5'-CTA GGC AAG GAA GGG CAA AAA GT-3'. Detection of *KHCLP*, *SHEPHERD*, and *TUB* gene expression was performed in a similar manner with primers based on the annotated sequences corresponding to AGI numbers At4g24170 (*KHCLP*), At4g24190 (*SHEPHERD*) and At5g44340 (*TUB*): SHEPHERD-FOR, 5'-CTG ATG GAT TAC GAA GAC AAA AAG-3'; SHEPHERD-REV, 5'-GTA AAC ACA AAG GAG AAA CAT CAG-3'; KHCLP-FOR, 5'-CTA AAA GCC TAA GTG GAG TGC-3'; KHCLP-REV, 5'-GCG GAG AAG AGA AGA TAT TGG-3' and TUB-FOR 5'-AAT ACG TCG GCG ATT CTC CG-3'; TUB-REV, 5'-CAC AGA CAC TGG ACT TGA CG-3'. For RT-PCR detection of *GUS* mRNA, the following primers were used: GUS-1, 5'-AGA CTG TAA CCA CGC GTC TC-3' and GUS-2, 5'-CCG ACA GCA GTT TCA TCA ATC-3' (Vroemen *et al.*, 1998).

AtTLP1 knockout mutant SGT5141

Knockout mutant SGT5141 (in *Ler-0* background) was selected from the database of gene trap lines carrying *Ds* element insertions that was previously described (Parinov *et al.*, 1999) and can be accessed via <http://www.plantcell.org/cgi/content/full/11/12/2263/DC1/1>. The *Ds* element in SGT5141 was found to be inserted 399 bp before the stop codon of the *AtTLP1* gene, leading to a truncated protein 133 amino acids shorter than the mature *AtTLP1* of 255 amino acids. Homozygous SGT5141 plants were tested for their ability to express rhizobacteria-mediated ISR as described above.

Results

Screening transposants for P. fluorescens WCS417r-induced GUS expression

In total 200 gene trap (WGT) transposants and 400 enhancer trap (WET) transposants were examined for *GUS* expression patterns in roots and leaves after colonization of the roots by ISR-inducing WCS417r bacteria. The gene trap lines were constructed to detect expression of a chromosomal gene with a transposon insertion within

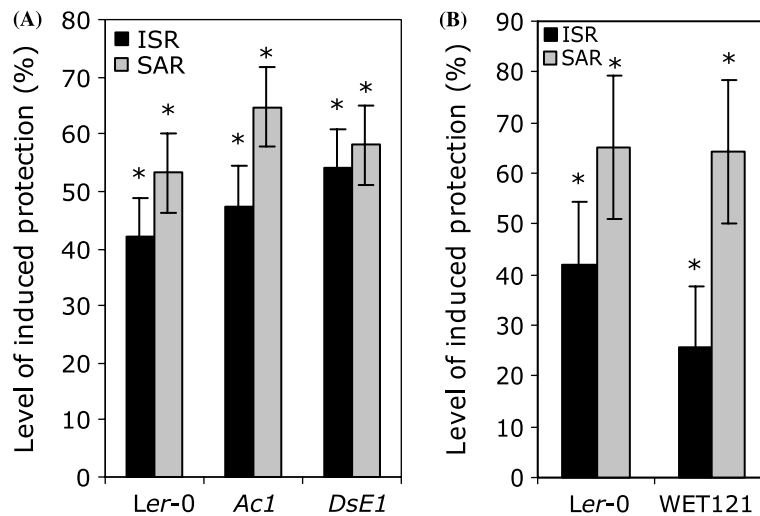


Figure 1. Levels of induced protection against *Pst* DC3000 as a result of *P. fluorescens* WCS417r-mediated ISR and *Pst* DC3000(*avrRpt2*)-induced SAR in wild-type *Ler-0* plants, the starter lines *Ac1* and *DsE1* (A) and enhancer trap line WET121 (B). ISR was induced by growing plants for 3 weeks in soil containing ISR-inducing *P. fluorescens* WCS417r bacteria at 5×10^7 cfu g⁻¹. Five-week-old plants were challenge inoculated with a bacterial suspension of virulent *Pst* DC3000 at 2.5×10^7 cfu ml⁻¹. Induction of SAR was performed 3 days before challenge inoculation by pressure infiltrating three lower leaves with a suspension of *Pst* DC3000(*avrRpt2*) bacteria at 10^7 cfu ml⁻¹. Four days after challenge inoculation, the percentage of diseased leaves was determined and the level of induced protection calculated on the basis of the reduction in the percentage of diseased leaves per plant relative to challenged control plants (set at 100%). The absolute proportions of diseased leaves of control-treated *Ler-0*, *Ac1*, and *DsE1* in (A) and *Ler-0* and WET121 in (B) were 61.9, 48.4, 48.2, 69.5, and 57.7, respectively. Asterisks indicate statistically significant differences compared to non-induced, *Pst* DC3000-challenged control plants (students *t*-test; $\alpha = 0.05$; $n = 20$). Data presented are means from a representative experiment that was repeated at least twice with similar results. Error bars represent standard errors.

the transcribed region. For this purpose, the *Ds* element contained a promoterless *GUS* gene, whose expression relies on the transcription of the chromosomal gene in which it is inserted (Sundaresan *et al.*, 1995; Vroemen *et al.*, 1998). The enhancer trap lines contained a *Ds* element with the *GUS* gene fused to a minimal -1 to -46 bp CaMV 35S promoter, which is not active in the absence of enhancer sequences. In these lines, expression of the *GUS* reporter gene is dependent on insertion near chromosomal enhancer sequences (Sundaresan *et al.*, 1995; Vroemen *et al.*, 1998). Thus, when the *Ds* element of an enhancer trap line is inserted in the proximity of a chromosomal gene, within or outside the coding sequence, *GUS* gene expression can be activated.

The WGT and WET transposants were constructed in the *Ler-0* background, an accession previously shown to develop ISR after colonization of the roots by WCS417r (Ton *et al.*, 1999; Van Wees *et al.*, 1997). Before screening the transposants, starter lines *Ac1* and *DsE1* (Vroemen *et al.*, 1998) were checked for their ability to express induced resistance against the

bacterial pathogen *Pst* DC3000 using standard ISR and SAR bioassays (Pieterse *et al.*, 1996). Figure 1A demonstrates that both starter lines were able to express levels of WCS417r-mediated ISR and *Pst* DC3000(*avrRpt2*)-induced SAR that are similar to those observed in wild-type *Ler-0* plants, indicating that the ability to express induced resistance was not affected in these lines.

To screen transposants for *GUS* expression patterns, 2-week-old seedlings of the WGT and WET lines were transplanted into soil with or without ISR-inducing WCS417r bacteria. Systemic protection in ISR-expressing plants results from the continuous stimulation of the plant by ISR-inducing rhizobacteria at the root surface. In *Arabidopsis*, full expression of WCS417r-mediated ISR in the leaves can be detected within 7 days after treatment of the roots with this rhizobacterial strain (Ton *et al.*, 2002b). All lines were harvested 4 and 11 days after induction treatment and examined for *GUS* expression patterns. Of the WGT lines, approximately 20% showed *GUS* expression, whereas approximately 35% of the WET lines stained for *GUS* activity. This is in

good agreement with the percentages found by Vroemen *et al.* (1998). Only in enhancer trap line WET121 *GUS* expression was specifically induced by the WCS417r treatment. In this line, specific *GUS* activity was apparent in the roots 4 days after colonization by WCS417r bacteria (Figures 2A–B). Interference contrast microscopy revealed that WCS417r-induced *GUS* activity was localized in the root vascular bundle (Figure 2C). No *GUS* activity was detected in leaves or reproductive organs (data not shown), indicating that the observed WCS417r-induced *GUS* expression was root specific.

Specificity of rhizobacteria-induced GUS expression in WET121

Development of rhizobacteria-mediated ISR is dependent on the plant/rhizobacterium combination, suggesting that recognition of the rhizobacteria by the plant is involved in the induction of ISR. Previously, rhizobacterial strains *P. fluorescens* WCS374r and *Pseudomonas putida* WCS358r were demonstrated to trigger ISR in radish and *Arabidopsis*, respectively (Leeman *et al.*, 1995; Van Wees *et al.*, 1997). To investigate whether these strains also activate *GUS* expression in WET121, they were applied to the roots of WET121 plants that were grown on vertical agar plates. *Escherichia coli* S17 bacteria that colonize *Arabidopsis* roots but do not induce ISR, were used as a control. Figures 2D–F show that WCS358r and WCS374r induced a similar *GUS* expression pattern in WET121 as did WCS417r. Application of *E. coli* bacteria did not result in *GUS* expression. Application of the *Pseudomonas* and *E. coli* bacterial strains to roots of soil-grown plants supported these findings (data not shown). The observation that *E. coli* strain S17 did not activate *GUS* expression suggests that physical colonization of the roots by bacteria *per se* is not sufficient to activate the *GUS* reporter gene in WET121. Apparently, specific components of the *Pseudomonas* rhizobacteria are required for the activation of the gene.

Chemical induction of GUS expression in WET121

WCS417r-mediated ISR in *Arabidopsis* functions independently of SA but requires responsiveness to the plant hormones JA and ET (Pieterse *et al.*,

1996, 1998). To investigate whether *GUS* expression in WET121 is regulated also by these signals, seedlings were grown on MS-agar containing 0.01 mM methyl jasmonate (MeJA), 0.1 mM of the ET precursor ACC, or 0.1 mM SA. The concentrations used were previously demonstrated to activate JA-, ET-, or SA-responsive gene expression, respectively, and to induce resistance against *Pst* DC3000 (Van Wees *et al.*, 1999). Figures 2G–I show that MeJA and SA did not induce *GUS* expression in WET121 seedlings. However, when incubated in the presence of ACC, WET121 seedlings showed a pronounced *GUS* expression pattern in the root vascular bundle, similar to that observed in rhizobacteria-treated WET121 plants (Figure 2J). These results indicate that the *Ds* element in WET121 is inserted in the vicinity of a rhizobacteria-inducible, ET-responsive gene.

Molecular analysis of WET121

To identify the gene activated in response to rhizobacteria and ACC, the number of *Ds* elements in the genomic DNA of WET121 was determined. Genomic DNA of WET121 and wild-type *Ler-0* plants was digested with *Pst*I, which cuts once in the *Ds* element outside of the *GUS* gene. Southern blot analysis using the *GUS* coding sequence as a probe, indicated that WET121 carried a single copy of the *Ds* element (Figure 3A).

To determine the location of the *Ds* element in the genome of WET121, genomic DNA flanking the *Ds* insertion was amplified by thermal asymmetric interlaced (TAIL) PCR, essentially as described previously (Liu *et al.*, 1995; Vroemen *et al.*, 1998). Figure 3B shows that TAIL-PCR resulted in specific PCR products in the secondary and tertiary round of TAIL-PCR. Sequencing of the flanking DNA revealed that the *Ds* element was inserted into chromosome 4 (Figure 3C). As is the case for most of the enhancer trap lines (Vroemen *et al.*, 1998), the *Ds* element was not inserted into an open reading frame. Thus, the rhizobacteria- and ACC-induced *GUS* expression was the result of the activity of (an) enhancer sequence(s) in the vicinity of the *Ds* element. Candidate genes in close proximity to the *Ds* element were a gene encoding a thaumatin-like protein (*AtTLPI*; At4g24180), a gene encoding a

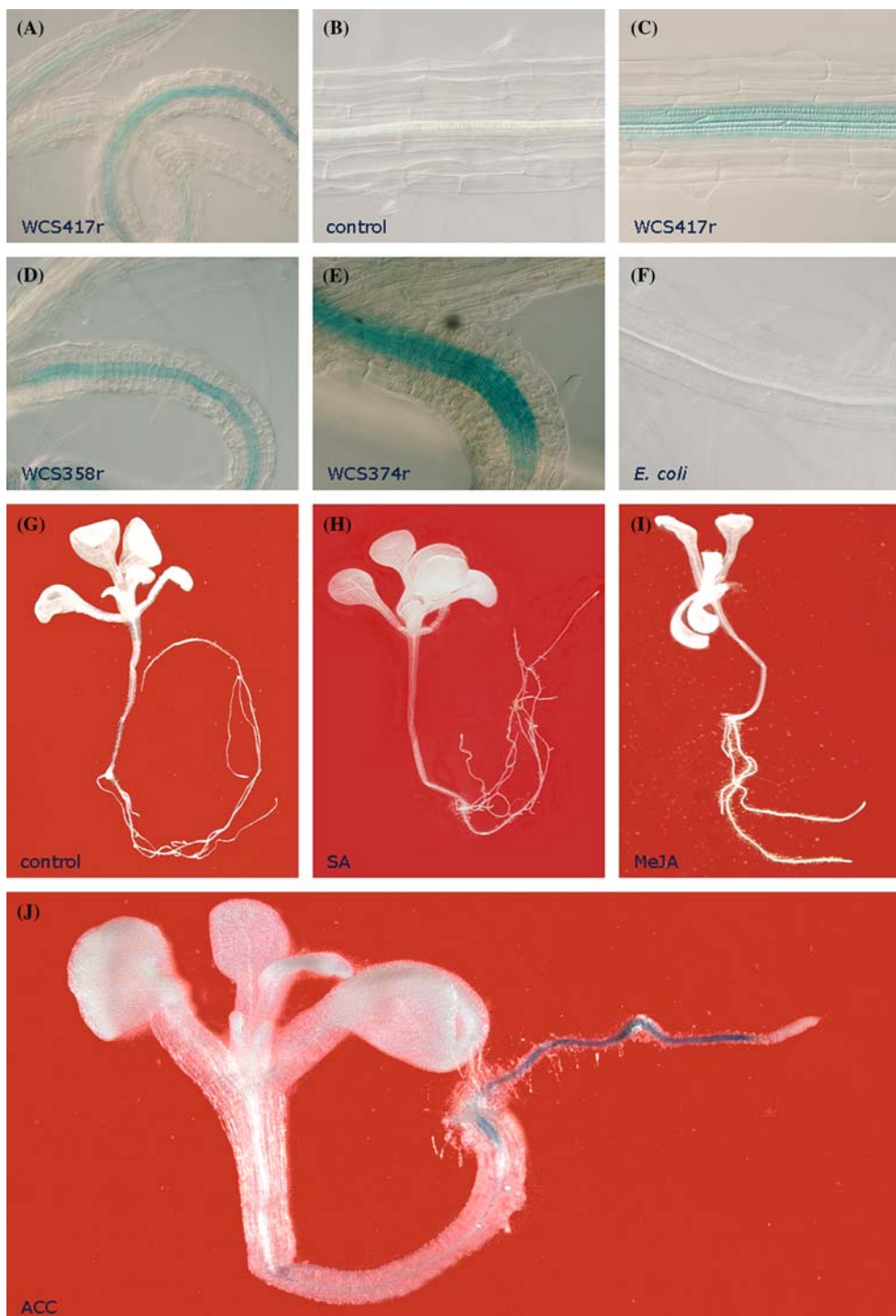


Figure 2. GUS expression patterns in enhancer trap line WET121. Pictures show light microscopic images (A, D–F), interference contrast microscopic images (B–C), or photographs (G–J) of roots grown in soil with *P. fluorescens* WCS417r (A + C); roots grown in soil without rhizobacteria (B); roots grown on MS-agar with *P. putida* WCS358r (D); roots grown on MS-agar with *P. fluorescens* WCS374r (E); roots grown on MS-agar with *E. coli* S17 (F); seedling grown on MS-agar (G) with 0.1 mM SA (H), 0.01 mM MeJA (I), or 0.1 mM ACC (J).

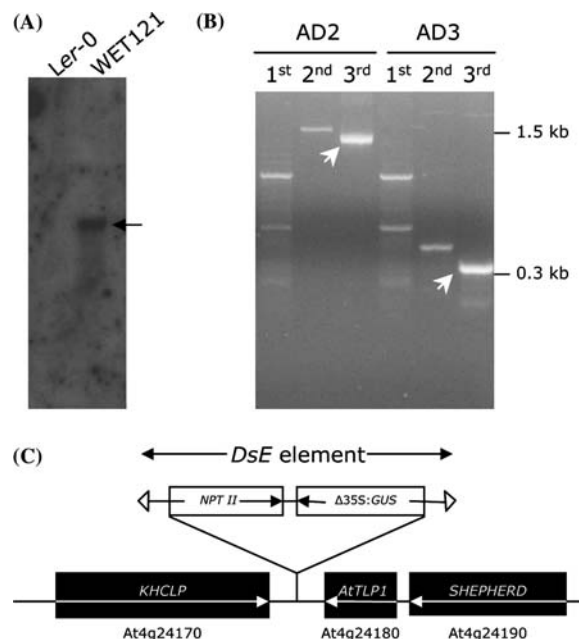


Figure 3. Molecular analysis of enhancer trap line WET121. (A) Southern blot of 10 μ g of genomic DNA from Ler-0 and WET121. The DNA was digested with *Pst*I, blotted and hybridized with the entire *GUS* coding sequence. (B) Ethidium bromide-stained agarose gel showing the TAIL-PCR products after the primary (1st), the secondary (2nd), and the tertiary (3rd) round of TAIL-PCR with arbitrary degenerated primers AD2 and AD3, and nested *Ds* specific primers Ds5-1 (1st), Ds5-2 (2nd), and Ds5-3 (3rd). In the secondary round of TAIL-PCR a specific PCR product appeared with both the AD2 and the AD3 primer combinations. These specific PCR products were further amplified in the tertiary round of TAIL-PCR, yielding PCR products of ca. 1.5 kb (with AD2) and 0.3 kb (with AD3). The PCR products in the tertiary round are shorter than those in the secondary round and are conform the predicted size reductions, indicating that the flanking sequences of the *Ds* element in WET121 were amplified successfully. Sequencing of these TAIL-PCR products revealed the flanking sequences of the *Ds* element insertion in WET121. (C) Schematic representation of the flanking sequences of the *Ds* element in WET121. Genes in the vicinity of the *Ds* element insertion encode a kinesin heavy chain-like protein (*KHCLP*), a thaumatin-like protein (*AtTLP1*) and the HSP90 type protein *SHEPHERD*.

kinesin heavy chain-like protein (*KHCLP*; At4g24170), and *SHEPHERD* (At424190) encoding a HSP90 type protein (Ishiguro *et al.*, 2002).

Because the *Ds* element in WET121 was not inserted in, or in the immediate vicinity of an open-reading frame, it was expected that WET121 would show normal ISR in response to colonization of the roots by WCS417r. Figure 1B shows that indeed, WET121 developed normal levels of ISR against *Pst* DC3000.

GUS and *AtTLP1* are co-regulated in WET121

Insertions in enhancer trap lines result in *GUS* expression when the *Ds* element is inserted either upstream or downstream of an active gene. Enhancers are known to act in an orientation-independent manner. Thus, insertions in either orientation can result in *GUS* expression (Vroemen *et al.*, 1998). To identify the gene that is responsible for the WCS417r- and ACC-induced activation of the *GUS* gene in WET121, the expression of *SHEPHERD*, *AtTLP1*, and *KHCLP* in control and ACC-treated WET121 plants was analyzed. Northern blot analysis showed that *SHEPHERD* is expressed in roots and leaves of both control- and ACC-treated plants (Figure 4A). This was incompatible with the WCS417r- and ACC-induced *GUS* expression pattern observed in WET121 and disqualified *SHEPHERD* as a candidate gene for co-regulation of the *GUS* gene.

KHCLP and *AtTLP1* transcripts could not be detected by northern blot analysis. Therefore, their expression patterns were examined by RT-PCR. Again, *KHCLP* mRNA could not be detected in roots of control-, WCS417r-, and ACC-treated plants (data not shown). Therefore, *AtTLP1* was the only candidate gene left in the close vicinity of the inserted *Ds* element that may be responsible for the vascular tissue-specific expression of the *GUS* gene in WCS417r- or ACC-treated roots. Indeed, *AtTLP1* transcripts accumulated in the roots upon treatment with ACC, which clearly correlated with the concomitant increase in *GUS* mRNA levels (Figure 4B). In the roots of untreated control plants, no *AtTLP1* mRNA could be detected. Moreover, both *AtTLP1* and *GUS* transcripts could not be detected in the leaves of control- and ACC-treated plants (data not shown). The strong correlation between the root-specific expression of *AtTLP1* and *GUS* strongly suggest that *GUS* expression observed in the root vascular bundle of WET121 is regulated by an enhancer of the *AtTLP1* gene and identifies the *AtTLP1* gene as a plant gene that is activated in the roots by ACC and fluorescent *Pseudomonas* spp.

AtTLP1 encodes a PR-S type protein

The *AtTLP1* gene encodes a thaumatin-like protein belonging to the PR-5 family of pathogenesis

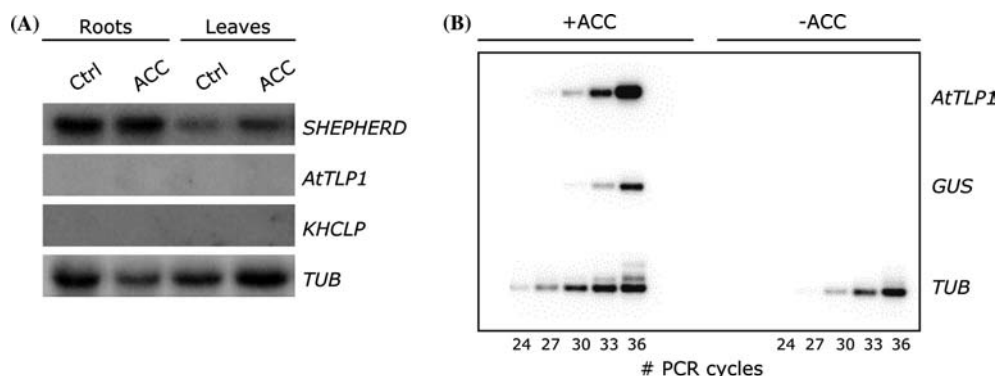


Figure 4. Expression analysis of genes flanking the *Ds* element insertion in WET121. Seedlings of WET121 were grown vertically on MS-agar plates for 12 days. Subsequently, 2 ml of water (Ctrl) or a solution containing 1 mM ACC was added onto the plates. Six days later, roots and leaves were harvested separately for RNA extraction. (A) Northern blot analysis of *SHEPHERD*, *AtTLP1*, and *KHCLP* mRNA in roots and leaves of WET121 plants grown with or without ACC. The blot was hybridized with gene-specific probes and stripped between hybridisations. To check for equal loading, the blot was hybridized with a gene-specific probe for β -tubulin (*TUB*). (B) RT-PCR analysis of *AtTLP1*, *GUS*, and *TUB* transcript levels in control- and ACC-treated roots of WET121. Shown are PCR products in the RT-PCR mixture after 24–36 PCR cycles (negative photograph of ethidium bromide-stained agarose gel).

related proteins. The putative AtTLP1 protein contains 255 amino acids and has a predicted molecular mass of 26.9 kD and a theoretical pI of 4.69 (<http://mips.gsf.de/proj/thal/db/>). Comparison of the predicted amino acid sequence with sequences in the databank revealed that it shares highest homology with the predicted amino acid sequence of the thaumatin-like isolog gene *AtTLI* (At4g38660; 70% identity) (Figure 5). AtTLP1 shares 56% identity at the amino acid level with the previously identified *Arabidopsis* AtPR-5 protein that is associated with SAR (Uknes *et al.*, 1992), and is homologous to other proteins belonging to the PR-5 family, including tobacco NtOsmotin (Singh *et al.*, 1989) (40% identity) and the tomato LePR-5x protein (39% identity), which was found to accumulate in the xylem sap of *F. oxysporum*-infected tomato plants (Rep *et al.*, 2002). The homology with the previously characterized PR-5 type proteins NtOsmotin and LePR-5x is relatively low. Figure 6 shows a phylogenetic tree of all 24 *Arabidopsis* PR-5 type proteins and selected PR-5 type proteins from other plant species. Within this tree, AtTLP1 clusters together with 7 other PR-5 type proteins of *Arabidopsis*, some of which have not been characterized so far. To investigate whether the eight genes of the *AtTLP1* cluster may have redundant functions, we analyzed their expression profile using the *Arabidopsis* microarray database and analysis toolbox Genevestigator (Zimmermann *et al.*, 2004). With

this toolbox it is possible to study how genes are co-expressed over selected microarrays in the database, and to which specific biotic and abiotic stress(es) the genes of interest are responsive. Comparison of the expression pattern of *AtTLP1* with that of the seven other PR-5-like genes (i.e. At1g20030, At1g75800, At2g17860, At4g36000, At4g36010, At4g38660, At4g38670) in 1434 Affymetrix 25k ATH1 GeneChip hybridization experiments, revealed that none of the 7 PR-5-like genes tested was co-expressed with *AtTLP1* (data not shown). While *AtTLP1* expression was only detectable in the roots, the other seven genes were either expressed in other tissues, or were responsive to biotic or abiotic stresses.

Effect of AtTLP1 knockout mutation

Previously, Parinov *et al.* (1999) generated a randomly mutagenized collection of gene trap lines carrying *Ds* element insertions throughout the *Arabidopsis* genome. This collection contained a knockout mutant line (SGT5141) with a *Ds* element inserted 133 bp upstream of the stop codon of the *AtTLP1* gene, leading to a truncated protein. The presence of the insertion was verified by TAIL-PCR as described for the WET121 line (data not shown). To examine the effect of this *AtTLP1* knockout mutation on the effectiveness of WCS417r-mediated ISR, ISR bioassays were performed with wild-type *Ler-0* and mutant SGT5141

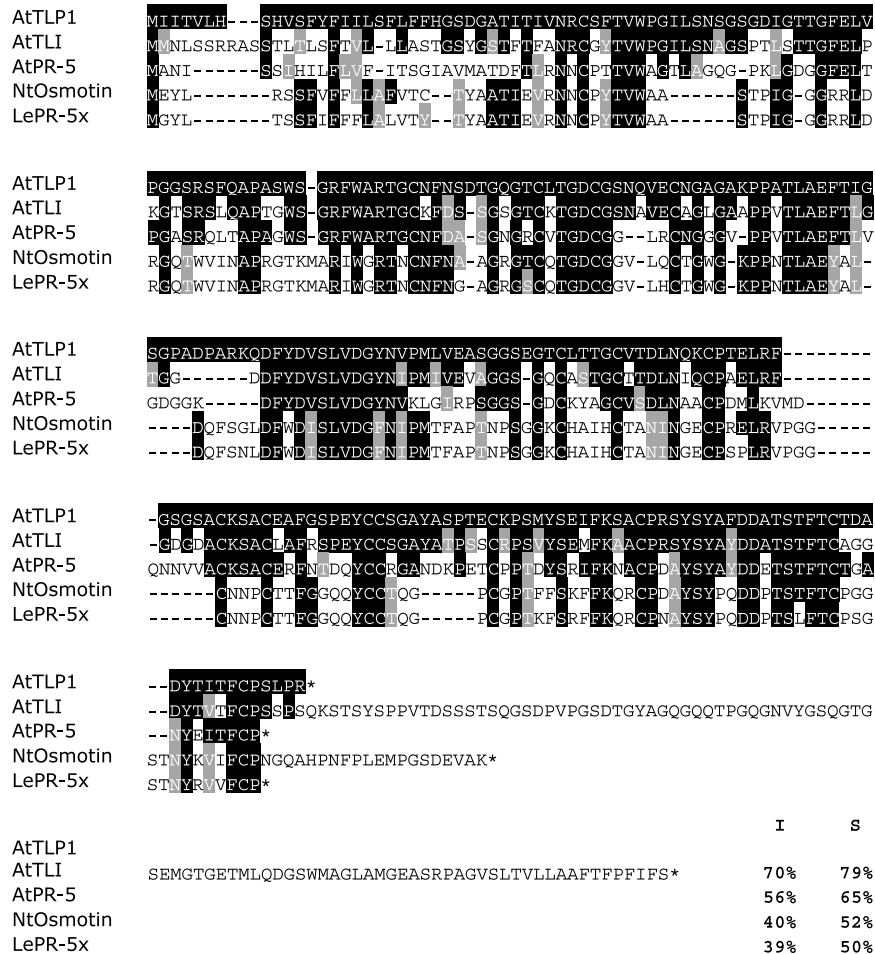


Figure 5. Comparison of the predicted amino acid sequence of AtTLP1 with other PR-5 type proteins. The alignment shows the amino acid sequences of several PR-5 type proteins. Sequences are from Arabidopsis AtTLP1 (AGI number At4g24180), Arabidopsis AtPR-5 (AGI number At1g75040; Uknes et al. (1992)), Arabidopsis AtTLI (AGI number At4g38660), tobacco NtOsmotin (GeneBank accession number X95308; Singh et al. (1989)), and LePR-5x (GeneBank accession number AAM23272; Rep et al. (2002)). Numbers above the alignments correspond to amino acid positions in AtTLP1. Black shading indicates amino acids identical to the AtTLP1 template, and gray shading indicates amino acids that are similar to the AtTLP1 template.

plants. For comparison, the effectiveness of pathogen-induced SAR was also examined. Figure 7 shows that SGT5141 developed levels of SAR similar to wild-type *Ler-0*. Moreover, SGT5141 showed the same level of ISR after colonization of the roots by WCS417r as did wild-type plants. Thus, the *Ds* insertion within the *AtTLP1* gene had no effect on the ability of SGT5141 to express ISR.

Discussion

Colonization of the roots of *Arabidopsis* by selected strains of fluorescent *Pseudomonas* spp.

leads to an enhanced defensive capacity that is effective against a broad range of foliar pathogens (Pieterse *et al.*, 2002). In contrast to pathogen-induced SAR, rhizobacteria-mediated ISR is not associated with induced expression of *PR* genes (Pieterse *et al.*, 1996, 1998; Van Wees *et al.*, 1999, 1997; Verhagen *et al.*, 2004). In search for genes that are specifically activated during ISR, we used a *Ac/Ds*-based gene/enhancer trap system, developed according to Sundaresan *et al.* (1995) by Vroemen *et al.* (1998), to detect genes that are specifically expressed in response to colonization of the roots by ISR-inducing WCS417r bacteria. Based on GUS activity staining, we identified an enhancer trap line that shows specific GUS

expression in the vascular bundle of *Arabidopsis* roots upon colonization by fluorescent *Pseudomonas* spp. strains WCS417r, WCS358r, and WCS374r, but not after colonization of the roots by ISR-non-inducing *E. coli* bacteria (Figure 2). These results indicate that colonization of the roots by bacteria *per se* is not sufficient to activate the *GUS* reporter gene in WET121. Apparently, specific components of the *Pseudomonas* rhizobacteria are required for the activation of the gene. The ET precursor ACC specifically activated the *GUS* gene as well (Figure 2). The specific ACC-induced activation of the *GUS* gene in vascular bundle of the roots closely resembled the *GUS* expression pattern induced by the rhizobacteria. It can, thus, be concluded that the *GUS* gene on the *Ds* element in WET121 is inserted in the vicinity of a rhizobacteria-inducible, ET-responsive gene.

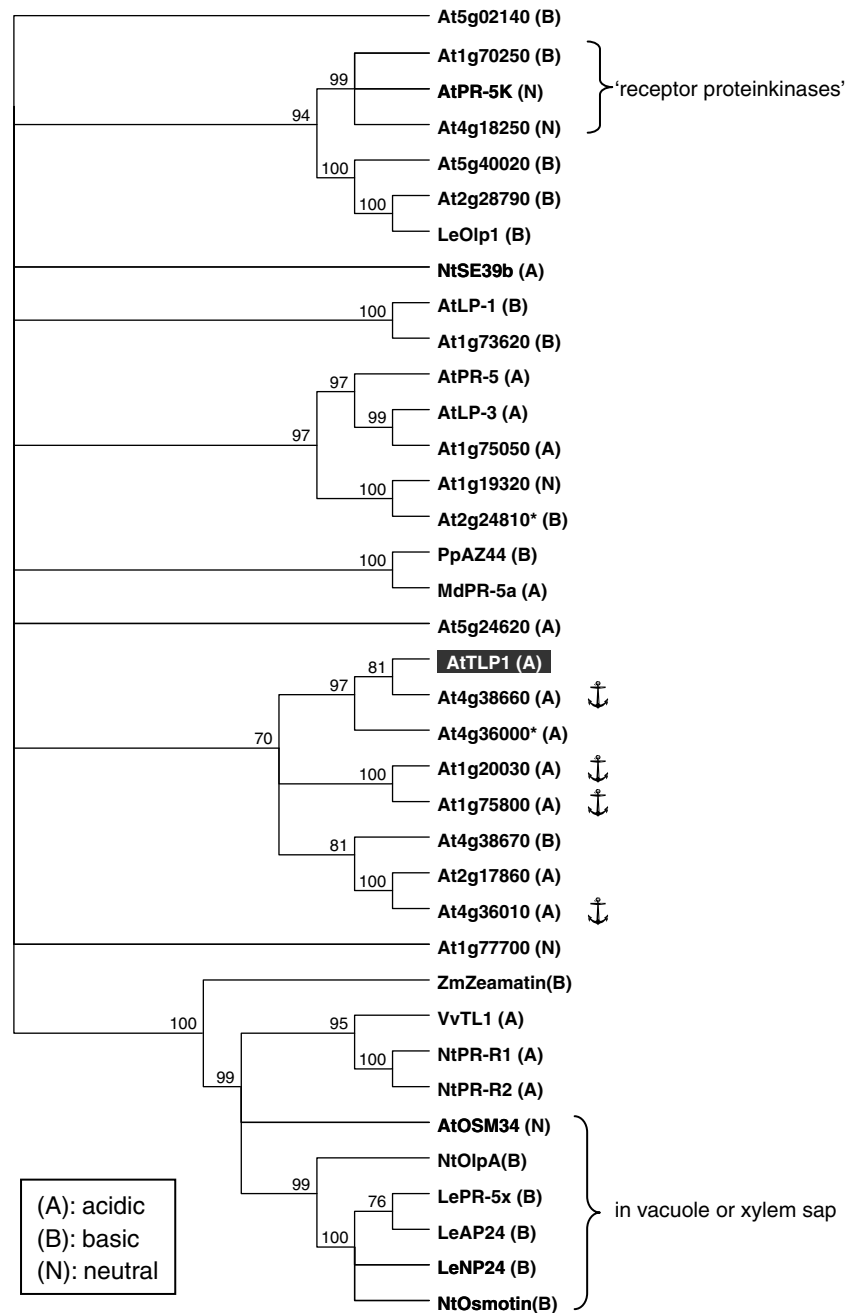
The *GUS* reporter gene in the *Ac/Ds*-transposon based gene/enhancer trap system used in this study responds to *cis*-acting transcriptional signals at the site of integration. These *cis*-acting transcriptional signals predominantly come from a gene in the close vicinity of the inserted *Ds* element (Ramachandran and Sundaresan, 2001). The *AtTLPI* gene, encoding a thaumatin-like protein that belongs to the evolutionarily conserved class of PR-5 proteins (Van Loon and Van Strien, 1999), was the only candidate gene that was coordinately activated with the *GUS* reporter gene in response to ACC treatment (Figures 3 and 4). Moreover, like the *GUS* reporter gene, *AtTLPI* showed a root-specific expression pattern in response to ACC treatment. These results indicate that the *GUS* reporter gene in transposant WET121 is regulated by an enhancer of the *AtTLPI* gene and identifies the *AtTLPI* gene as a plant gene that is activated in the vascular bundle of *Arabidopsis* roots by ACC and fluorescent *Pseudomonas* spp.

Rhizobacterial strains WCS417r, WCS358r, and WCS374r all induced the expression of the *GUS* reporter gene in WET121. In the case of WCS417r and WCS358r this nicely correlates with their ability to trigger ISR in *Arabidopsis* (Van Wees *et al.*, 1997). However, strain WCS374r, which consistently induces ISR in radish (Leeman *et al.*, 1995), but shows variable results in *Arabidopsis* (Van Wees *et al.*, 1997; Ran *et al.*, 2000), activated the *GUS* reporter gene in WET121 as well. Therefore, these results did not allow a

conclusion concerning the importance of the concomitantly expressed *AtTLPI* gene in the onset or expression of ISR. To gain insight in the role of *AtTLPI* in the onset of ISR, *AtTLPI* knockout mutant plants were tested on their ability to express WCS417r-induced ISR. Knockout mutant plants (Figure 7), as well as *AtTLPI*-overexpressing transgenic plants (data not shown) developed normal levels of WCS417r-induced ISR against *Pst* DC3000, indicating that the rhizobacteria-induced *AtTLPI* gene is unlikely to play a crucial role in ISR against this foliar pathogen.

Previously, Hase *et al.* (2003) described another response of *Arabidopsis* to *Pseudomonas* bacteria that seems to be unrelated to the ability to induce ISR. Leaves of plants of which the roots were colonized by WCS417r, WCS358r, or WCS374r bacteria showed an enhanced capacity to convert the ET precursor ACC to ET, leading to a potentiated expression of the ET-responsive genes *PDFI.2* and *HEL* after treatment of the leaves with 1 mM ACC, and a significantly higher level of ET emission after pathogen challenge. This enhanced ACC-converting capacity was also apparent in the ISR-impaired mutants *jar1-1* and *npr1-1* after treatment of the roots with *Pseudomonas* bacteria. These induced plant responses to colonization of the roots by non-pathogenic *Pseudomonas* bacteria demonstrate that *Arabidopsis* plants perceive signals from the rhizobacteria that are present on the root surface, and translate these signals into local and systemic responses. The exact role of these rhizobacteria-induced plant responses in induced resistance or other plant growth-promoting characteristics that are associated with these plant-PGPR interactions, remains to be further elucidated.

The *AtTLPI* gene encodes a thaumatin-like protein, which belongs to the evolutionarily conserved class of PR-5 proteins (Van Loon and Van Strien, 1999). A large number of PR-5 proteins from different plant species has been isolated and characterized (Anžlovar and Dermastia, 2003), all of which show similarity to the sweet-tasting protein thaumatin present in the fruits of the tropical shrub *Thaumatococcus daniellii* (Van der Wel and Loeve, 1972). Several PR-5 proteins have been shown to possess antimicrobial activity *in vitro* (Woloshuk *et al.*, 1991; Anžlovar and Dermastia, 2003), or *in vivo* (Liu *et al.*, 1994), or appear to be involved in osmotic adaptation



(Singh *et al.*, 1987; Anžlovar and Dermastia, 2003). Besides constitutive and tissue-specific *PR-5* genes, many *PR-5* genes are induced upon biotic or abiotic stresses (Van Loon and Van Strien, 1999). Among the 24 putative *PR-5* proteins in *Arabidopsis*, *AtTLPI* clusters together with 7, so far uncharacterized homologues (Figure 6). Comparison of expression profiles of the eight *PR-5*-

like genes in the *AtTLPI* cluster showed that *AtTLPI* is not co-expressed with any of the seven homologues in the cluster, suggesting that the function of *AtTLPI* is not redundant. The amino acid sequence homology of *AtTLPI* with the previously characterized acidic *AtPR-5* protein (Uknes *et al.*, 1992) is relatively low (56% identity), suggesting that the two proteins have different

functions. This is supported by the fact that the expression of *AtTLP1* is regulated by ET (Figures 2 and 4), whereas *AtPR-5* is controlled in a SA-dependent manner (Uknes *et al.*, 1992). Moreover, *AtPR-5* is not activated in response to colonization of the roots by ISR-inducing WCS417r bacteria (Pieterse *et al.*, 1996, 1998; Van Wees *et al.*, 1997). *AtTLP1* is specifically activated in the vascular bundle upon colonization of the roots by *Pseudomonas* bacteria. Previously, the tomato PR-5 type protein LePR-5x was found to accumulate in xylem sap of tomato plants after root infection with *F. oxysporum* (Rep *et al.*, 2002). However, in contrast to the putative AtTLP1 protein, LePR-5x is basic and has only low homology with AtTLP1 (39% identity). Thus, although both AtTLP1 and LePR-5x are predicted to act in the vascular bundle, it is not known whether they have a similar function.

What might be the role of induced *AtTLP1* gene expression in roots that are colonized by *Pseudomonas* bacteria? Most of the isolated PR-5 type proteins possess antimicrobial activity in *in vitro* assays, in which they inhibit germination and cause lysis of spores of fungal and oomycete pathogens (Woloshuk *et al.*, 1991; Abad *et al.*, 1996). Although the specific mode of action of PR-5 type proteins is not known, they are thought to be capable of permeabilizing fungal membranes

(Anžlovar and Dermastia, 2003). Because *AtTLP1* is specifically expressed in the root vascular bundle, the putative AtTLP1 protein may have a local antimicrobial function.

Transposons have been used extensively for insertional mutagenesis of several plant species in order to identify and characterize genes conferring a mutant phenotype (Ramachandran and Sundaresan, 2001). A useful aspect of the *Ac/Ds*-transposon based gene/enhancer trap system described by Sundaresan *et al.* (1995) is that the transposons carry a *GUS* reporter gene that can respond to *cis* acting transcriptional signals at the site of integration, thereby facilitating the identification of genes based on their expression pattern, rather than on their mutant phenotype. Another advantage of this system is that it may allow the identification of genes that, due their low or restricted expression patterns, remain undetected in other differential screening methods or DNA microarray analyses. Despite the relatively small screen of 200 gene trap and 400 enhancer trap lines we were able to identify *AtTLP1* as an example of such a gene. The *GUS* gene in enhancer trap line WET121, which is activated in *cis* by the *AtTLP1* gene in the vicinity of the *Ds* insertion, was expressed in a tissue-specific manner in the roots in response to colonization by non-pathogenic rhizobacteria. Rhizobacteria-induced expression

←
Figure 6. Phylogenetic tree of Arabidopsis PR-5 proteins and selected PR-5 proteins from other plant species. All 24 predicted proteins of Arabidopsis with a PR-5 ("thaumatin-like") domain present in The Arabidopsis Information Resource (www.arabidopsis.org) were aligned with selected PR-5 proteins from other plant species. Additions to the core PR-5 consensus sequence at N-termini (signal sequences) and C-termini (vacuolar targeting sequences or other extensions beyond the conserved sequence "FCP") were trimmed. This alignment was used to construct a phylogenetic tree using Neighbour-Joining. A PR-5-like protein from *Caenorhabditis elegans* (accession AAF60831) was used to root the tree. Based on the pI of the core sequences, the proteins were classified as acidic (pI < 5.5, "A"), neutral (5.5 < pI < 7.5, "N") or basic (pI > 7.5, "B"). Four Arabidopsis PR-5 proteins have long C-terminal extensions (71–100 residues) ending with a stretch of about 20 hydrophobic residues that could serve as a membrane anchor (e.g. AtTLI; At4g38660 in Figure 5). In the figure, these are indicated with an anchor. Three Arabidopsis proteins that have a central hydrophobic region and a C-terminal protein kinase domain characteristic of receptor protein kinases (Wang *et al.*, 1996) group together based on their PR-5 core sequence. Published sequences are referred to by protein names: PR-5 (Uknes *et al.*, 1992), PR-5K (Wang *et al.*, 1996), OSM34 (Capelli *et al.*, 1997), LP-1 (Hu and Reddy, 1995), LP-3 (Hu and Reddy, 1997) from Arabidopsis; Olp1 (Chen *et al.*, 1996), NP24 (King *et al.*, 1988), AP24 (Ruiz-Medrano *et al.*, 1992) and PR-5x (Rep *et al.*, 2002) from tomato; Osmotin (Singh *et al.*, 1989), SE39b (Kuboyama *et al.*, 1997), PR-R1 (Payne *et al.*, 1988), and PR-R2 (Cornelissen *et al.*, 1986) from tobacco; Zeamatin (Richardson *et al.*, 1987) from maize; PpAZ44 (Ruperti *et al.*, 2002) from peach; MdPR-5a (Oh *et al.*, 2000) from apple; VvTL1 (Tattersall *et al.*, 1997) from grape. All sequence names are preceded by species abbreviations (At: Arabidopsis, Le: tomato, Md: apple, Nt: tobacco, Pp: peach, Vv: grape, Zm: maize). Bootstrap percentages are provided for branches receiving 70% or more support. Branches with less support were collapsed because in these cases branch sequence was considered to be too unreliable. Note: In two genes (At2g24810 and At4g36000) proposed introns were ignored to optimize the alignment of the respective translation products with the other proteins. Instead of the large internal deletions in coding sequences due to the presence of introns in the current gene models, this led to a single stop codon in the case of At4g36000 and an internal deletion of only 28 bp (thus a frameshift) in the case of At2g24810. Both genes are therefore likely to be pseudogenes and are marked with an asterisk (*) in the tree.

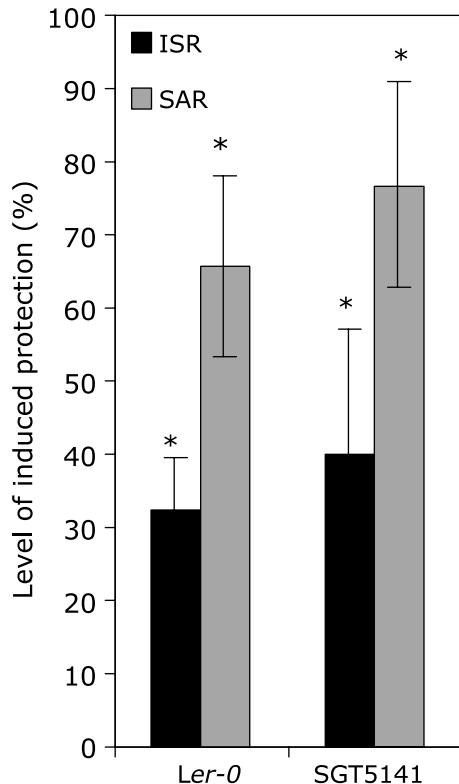


Figure 7. Levels of induced protection against *Pst* DC3000 as a result of *P. fluorescens* WCS417r-mediated ISR and pathogen-induced SAR in *Ler-0* and the *AtTLP1* knockout mutant SGT5141. Four days after challenge inoculation, the percentage of diseased leaves was determined and the level of induced protection calculated on the basis of the reduction in the percentage of diseased leaves per plant relative to challenged control plants (set at 100%). The absolute proportions of diseased leaves of control-treated *Ler-0* and SGT5141 plants were 44.0 and 32.3, respectively. For details see legend to Figure 1.

levels of *AtTLP1* were below the detection limit of Affymetrix GeneChip hybridizations when total RNA from roots was used as template for probe preparations (Verhagen *et al.*, 2004). Thus, screening of the *Ac/Ds*-based gene/enhancer trap system can have an added value when compared to other methods for gene discovery.

Acknowledgements

We thank Dr Casper Vroemen for kindly providing the gene trap and enhancer trap lines, and Martin de Vos and Sjoerd van der Ent for critically reading the manuscript. This research was

supported, in part, by grant BIO4-CT97-2120 of the European Commission Fourth Framework.

References

- Abad, L.R., Paino D'Urzo, M., Lin, D., Narasimhan, M.L., Renveni, M., Zhu, J.K., Niu, X., Singh, N.K., Hasegawa, P.M. and Bressan, R.A. 1996. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. *Plant Sci.* 118: 11–23.
- Anžlovar, S. and Dermastia, M. 2003. The comparative analysis of osmotins and osmotin-like PR-5 proteins. *Plant Biol.* 5: 116–124.
- Bigirimana, J. and Höfte, M. 2002. Induction of systemic resistance to *Colletotrichum lindemuthianum* in bean by a benzothiadiazole derivative and rhizobacteria. *Phytoparasitica* 30: 159–168.
- Capelli, N., Diogon, T., Greppin, H. and Simon, P. 1997. Isolation and characterization of a cDNA clone encoding an osmotin-like protein from *Arabidopsis thaliana*. *Gene* 191: 51–56.
- Chen, R., Wang, F. and Smith, A.G. 1996. A flower-specific gene encoding an osmotin-like protein from *Lycopersicon esculentum*. *Gene* 179: 301–302.
- Conrath, U., Pieterse, C.M.J. and Mauch-Mani, B. 2002. Priming in plant-pathogen interactions. *Trends Plant Sci.* 7: 210–216.
- Cornelissen, B.J., Hooft van Huijsduijnen, R.A. and Bol, J.F. 1986. A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. *Nature* 321: 531–532.
- De Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Métraux, J.-P. and Höfte, M. 1999. Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. *Mol. Plant-Microbe Interact.* 12: 450–458.
- Dong, X.N. 2004. NPR1, all things considered. *Curr. Opin. Plant Biol.* 7: 547–552.
- Duijff, B.J., Pouhair, D., Olivain, C., Alabouvette, C. and Lemanceau, P. 1998. Implication of systemic induced resistance in the suppression of fusarium wilt of tomato by *Pseudomonas fluorescens* WCS417r and by nonpathogenic *Fusarium oxysporum* Fo47. *Eur. J. Plant Pathol.* 104: 903–910.
- Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261: 754–756.
- Hase, S., Van Pelt, J.A., Loon, L.C. Van and Pieterse, C.M.J. 2003. Colonization of *Arabidopsis* roots by *Pseudomonas fluorescens* primes the plant to produce higher levels of ethylene upon pathogen infection. *Physiol. Mol. Plant Pathol.* 62: 219–226.
- Hoagland, D.R. and Arnon, D.I. 1938. The water culture method for growing plants without soil. *Calif. Agric. Exp. Stn. Bull.* 347: 36–39.
- Hu, X. and Reddy, A.S. 1995. Nucleotide sequence of a cDNA clone encoding a thaumatin-like protein from *Arabidopsis*. *Plant Physiol.* 107: 305–306.
- Hu, X. and Reddy, A.S. 1997. Cloning and expression of a PR5-like protein from *Arabidopsis*: inhibition of fungal growth by bacterially expressed protein. *Plant Mol. Biol.* 34: 949–959.

- Iavicoli, A., Boutet, E., Buchala, A. and Métraux, J.-P. 2003. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Mol. Plant-Microbe Interact.* 16: 851–858.
- Ishiguro, S., Watanabe, Y., Ito, N., Nonaka, H., Takeda, N., Sakai, T., Kanaya, H. and Okada, K. 2002. SHEPHERD is the *Arabidopsis* GRP94 responsible for the formation of functional CLAVATA proteins. *EMBO J.* 21: 898–908.
- King, E.O., Ward, M.K. and Raney, D.E. 1954. Two simple media for the demonstration of phycocyanin and fluorescein. *J. Lab. Clin. Med.* 44: 301–307.
- King, G.J., Turner, V.A., Hussey, C.E. Jr., Syrkin Wurtele, E. and Lee, M. 1988. Isolation and characterization of a tomato cDNA clone which codes for a salt-induced protein. *Plant Mol. Biol.* 10: 401–412.
- Klopper, J.W., Leong, J., Teintze, M. and Schroth, M.N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* 286: 885–886.
- Knoester, M., Pieterse, C.M.J., Bol, J.F. Van Loon, L.C. 1999. Systemic resistance in *Arabidopsis* induced by rhizobacteria requires ethylene-dependent signaling at the site of application. *Mol. Plant-Microbe Interact.* 12: 720–727.
- Kuboyama, T., Yoshida, K.T. and Takeda, G. 1997. An acidic 39-kDa protein secreted from stigmas of tobacco has an amino-terminal motif that is conserved among thaumatin-like proteins. *Plant Cell Physiol.* 38: 91–95.
- Kunkel, B.N., Bent, A.F., Dahlbeck, D., Innes, R.W. and Staskawicz, B.J. 1993. *RPS2*, an *Arabidopsis* disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene *AvrRpt2*. *Plant Cell* 5: 865–875.
- Leeman, M., Van Pelt, J.A., Den Ouden, F.M., Heinsbroek, M., Bakker, P.A.H.M. and Schippers, B. 1995. Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. *Eur. J. Plant Pathol.* 101: 655–664.
- Liu, D., Raghothama, K.G., Hasegawa, P.M. and Bressan, R.A. 1994. Osmotin overexpression in potato delays development of disease symptoms. *Proc. Natl. Acad. Sci. USA* 91: 1888–1892.
- Liu, Y.-G., Mitsukawa, N., Oosumi, T. and Whittier, R.F. 1995. Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* 8: 457–463.
- Maurhofer, M., Reimann, C., Schmidli-Sacherer, P., Heeb, S.D. and Défago, G. 1998. Salicylic acid biosynthesis genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. *Phytopathology* 88: 678–684.
- Nawrath, C., Heck, S., Parinthewong, N. and Métraux, J.-P. 2002. EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. *Plant Cell* 14: 275–286.
- Oh, D.H., Song, K.J., Shin, Y.U. and Chung, W.I. 2000. Isolation of a cDNA encoding a 31-kDa, pathogenesis-related 5/thaumatin-like (PR5/TL) protein abundantly expressed in apple fruit (*Malus domestica* cv. Fuji). *Biosci. Biotechnol. Biochem.* 64: 355–362.
- Parinov, S., Sevugan, M., Ye, D., Yang, W.C., Kumaran, M. and Sundaresan, V. 1999. Analysis of flanking sequences from *Dissociation* insertion lines: a database for reverse genetics in *Arabidopsis*. *Plant Cell* 11: 2263–2270.
- Payne, G., Middlesteadt, W., Williams, S., Desai, N., Parks, T.D., Dincher, S., Carnes, M. and Ryals, J. 1988. Isolation and nucleotide-sequence of a novel cDNA clone encoding the major form of pathogenesis-related protein-R. *Plant Mol. Biol.* 11: 223–224.
- Pieterse, C.M.J. and Van Loon, L.C. 2004. NPR1: the spider in the web of induced resistance signaling pathways. *Curr. Opin. Plant Biol.* 7: 456–464.
- Pieterse, C.M.J., Van Pelt, J.A., Ton, J., Parchmann, S., Mueller, M.J., Buchala, A.J., Métraux, J.-P. and Van Loon, L.C. 2000. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. *Physiol. Mol. Plant Pathol.* 57: 123–134.
- Pieterse, C.M.J., Van Wees, S.C.M., Hoffland, E., Van Pelt, J.A. and Van Loon, L.C. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* 8: 1225–1237.
- Pieterse, C.M.J., Van Wees, S.C.M., Ton, J., Van Pelt, J.A. and Van Loon, L.C. 2002. Signalling in rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *Plant Biol.* 4: 535–544.
- Pieterse, C.M.J., Van Wees, S.C.M., Van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. and Van Loon, L.C. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* 10: 1571–1580.
- Press, C.M., Wilson, M., Tuzun, S. and Klopper, J.W. 1997. Salicylic acid produced by *Serratiamarcescens* 91–166 is not the primary determinant of induced systemic resistance in cucumber or tobacco. *Mol. Plant-Microbe Interact.* 10: 761–768.
- Raaijmakers, J.M. and Weller, D.M. 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. *Mol. Plant-Microbe Interact.* 11: 144–152.
- Ramachandran, S. and Sundaresan, V. 2001. Transposons as tools for functional genomics. *Plant Physiol. Biochem.* 39: 243–252.
- Ran, L.X., Van Loon, L.C. and Bakker, P.A.H.M. 2000. No role for bacterially-produced salicylic acid in induction of systemic resistance in *Arabidopsis* suppression of bacterial wilt in *Eucalyptus* and bacterial speck in *Arabidopsis* by fluorescent *Pseudomonas* spp. strains: conditions and mechanisms. Ph.D. Thesis L.X. Ran, Utrecht University, pp. 70–82.
- Rep, M., Dekker, H.L., Vossen, J.H., de Boer, A.D., Houterman, P.M., Speijer, D., Back, J.W., de Koster, C.G. and Cornelissen, B.J.C. 2002. Mass spectrometric identification of isoforms of PR proteins in xylem sap of fungus-infected tomato. *Plant Physiol.* 130: 904–917.
- Richardson, M., Valdes-Rodriguez, S. and Blanco-Labra, A. 1987. A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. *Nature* 327: 432–434.
- Ross, A.F. 1961. Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14: 340–358.
- Ruiz-Medrano, R., Jimenez-Moraila, B., Herrera-Estrella, L. and Rivera-Bustamante, R.F. 1992. Nucleotide sequence of an osmotin-like cDNA induced in tomato during viroid infection. *Plant Mol. Biol.* 20: 1199–1202.
- Ruperti, B., Cattivelli, L., Pagni, S. and Ramina, A. 2002. Ethylene-responsive genes are differentially regulated during abscission, organ senescence and wounding in peach (*Prunus persica*). *J. Exp. Bot.* 53: 429–437.
- Ryu, C.-M., Hu, C.-H., Reddy, M.S. and Klopper, J.W. 2003. Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathogens of *Pseudomonas syringae*. *New Phytologist* 160: 413–420.

- Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
- Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C. and Manners, J.M. 2000. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proc. Natl. Acad. Sci. USA* 97: 11655–11660.
- Schippers, B., Bakker, A.W. and Bakker, P.A.H.M. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annu. Rev. Phytopathol.* 115: 339–358.
- Singh, N.K., Bracker, C.A., Hasegawa, P.M., Handa, A.K., Buckel, S., Hermodson, M.A., Pfankoch, E., Regnier, F.E. and Bressan, R.A. 1987. Characterization of osmotin. *Plant Physiol.* 85: 529–536.
- Singh, N.K., Nelson, D.E., Kuhn, D., Hasegawa, P.M. and Bressan, R.A. 1989. Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. *Plant Physiol.* 90: 1096–1101.
- Spoel, S.H., Koornneef, A., Claessens, S.M.C., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Métraux, J.-P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X. and Pieterse, C.M.J. 2003. NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15: 760–770.
- Sticher, L., Mauch-Mani, B. and Métraux, J.-P. 1997. Systemic acquired resistance. *Annu. Rev. Phytopathol.* 35: 235–270.
- Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J.D.G., Dean, C., Ma, H. and Martienssen, R. 1995. Patterns of gene-action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev.* 9: 1797–1810.
- Tattersall, D.B., Van Heeswijk, R. and Hoj, P.B. 1997. Identification and characterization of a fruit-specific, thaumatin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. *Plant Physiol.* 114: 759–769.
- Ton, J., De Vos, M., Robben, C., Buchala, A.J., Métraux, J.-P., Van Loon, L.C. and Pieterse, C.M.J. 2002a. Characterisation of *Arabidopsis* enhanced disease susceptibility mutants that are affected in systemically induced resistance. *Plant J.* 29: 11–21.
- Ton, J., Pieterse, C.M.J. and Van Loon, L.C. 1999. Identification of a locus in *Arabidopsis* controlling both the expression of rhizobacteria-mediated induced systemic resistance (ISR) and basal resistance against *Pseudomonas syringae* pv. *tomato*. *Mol. Plant–Microbe Interact.* 12: 911–918.
- Ton, J., Van Pelt, J.A., Van Loon, L.C. and Pieterse, C.M.J. 2002b. Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. *Mol. Plant–Microbe Interact.* 15: 27–34.
- Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J. 1992. Acquired resistance in *Arabidopsis*. *Plant Cell* 4: 645–656.
- Van der Wel, H. and Loeve, K. 1972. Isolation and characterization of Thaumatin 1 and 2, the sweet-tasting proteins from *Thaumatococcus daniellii* Benth. *Eur. J. Biochem.* 31: 221–225.
- Van Loon, L.C. and Bakker, P.A.H.M. 2003. Signalling in rhizobacteria-plant interactions. In: J. E.J.W. De Kroon Visser (Ed.), *Ecological Studies*, Springer-Verlag, Berlin Heidelberg, pp. 287–330.
- Van Loon, L.C., Bakker, P.A.H.M. and Pieterse, C.M.J. 1998. Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36: 453–483.
- Van Loon, L.C. and Van Strien, E.A. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* 55: 85–97.
- Van Peer, R., Niemann, G.J. and Schippers, B. 1991. Induced resistance and phytoalexin accumulation in biological control of fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. *Phytopathology* 91: 728–734.
- Van Wees, S.C.M., De Swart, E.A.M., Van Pelt, J.A., Van Loon, L.C. and Pieterse, C.M.J. 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 97: 8711–8716.
- Van Wees, S.C.M., Luijendijk, M., Smoorenburg, I., Van Loon, L.C. and Pieterse, C.M.J. 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. *Plant Mol. Biol.* 41: 537–549.
- Van Wees, S.C.M., Pieterse, C.M.J., Trijssenaar, A., Van Westende, Y.A.M., Hartog, F. and Van Loon, L.C. 1997. Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Mol. Plant–Microbe Interact.* 10: 716–724.
- Verhagen, B.W.M., Glazebrook, J., Zhu, T., Chang, H.-S., Van Loon, L.C. and Pieterse, C.M.J. 2004. The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Mol. Plant–Microbe Interact.* 17: 895–908.
- Vroemen, C.W., Aarts, N., In der Rieden, P.M.J., Van Kammen, A. and De Vries, S.C. 1998. Identification of genes expressed during *Arabidopsis thaliana* embryogenesis using enhancer trap and gene trap Ds-transposons. In: N.V. Raikhel (Ed.), *Cellular Integration of Signal Transduction Pathways*, Springer Verlag, Berlin, pp. 207–232.
- Wang, X., Zafian, P., Choudhary, M. and Lawton, M. 1996. The PR5K receptor protein kinase from *Arabidopsis thaliana* is structurally related to a family of plant defense proteins. *Proc. Natl. Acad. Sci. USA* 93: 2598–2602.
- Whalen, M.C., Innes, R.W., Bent, A.F. and Staskawicz, B.J. 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* 3: 49–59.
- Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414: 562–565.
- Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlage, P.M., Van den Elzen, P.J.M. and Cornelissen, B.J.C. 1991. Pathogen-induced proteins with inhibitory activity towards *Phytophthora infestans*. *Plant Cell* 3: 619–628.
- Yan, Z., Reddy, M.S., Ryu, C.-M., McNroy, J.A., Wilson, M. and Kloepper, J.W. 2002. Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. *Phytopathol.* 92: 1329–1333.
- Zhang, S., Moyne, A.-L., Reddy, M.S. and Kloepper, J.W. 2002. The role of salicylic acid in induced systemic resistance elicited by plant growth-promoting rhizobacteria against blue mold of tobacco. *Biol. Control* 25: 288–296.
- Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. and Gruissem, W. 2004. GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. *Plant Physiol.* 136: 2621–2632.