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The Transcriptome of Rhizobacteria-Induced Systemic Resistance in *Arabidopsis*

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Plants develop an enhanced defensive capacity against a broad spectrum of plant pathogens after colonization of the roots by selected strains of nonpathogenic, fluorescent *Pseudomonas* spp. In *Arabidopsis thaliana*, this rhizobacteria-induced systemic resistance (ISR) functions independently of salicylic acid but requires responsiveness to the plant hormones jasmonic acid and ethylene. In contrast to pathogen-induced systemic acquired resistance, rhizobacteria-mediated ISR is not associated with changes in the expression of genes encoding pathogenesis-related proteins. To identify ISR-related genes, we surveyed the transcriptional response of over 8,000 *Arabidopsis* genes during rhizobacteria-mediated ISR. Locally in the roots, ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria elicited a substantial change in the expression of 97 genes. However, systemically in the leaves, none of the approximately 8,000 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 *P. syringae* pv. *tomato* DC3000, 81 genes showed an augmented expression pattern in ISR-expressing leaves, suggesting that these genes were primed to respond faster or more strongly upon pathogen attack. The majority of the primed genes was predicted to be regulated by jasmonic acid or ethylene signaling. Priming of pathogen-induced genes allows the plant to react more effectively to the invader encountered, which might explain the broad-spectrum action of rhizobacteria-mediated ISR.

Additional keywords: transcript profiling.

Selected strains of root-colonizing, fluorescent *Pseudomonas* spp. have been shown to trigger a plant-mediated resistance response in aboveground plant parts. This type of induced resistance is referred to as rhizobacteria-mediated induced systemic resistance (ISR) (Van Loon et al. 1998). Phenotypically, rhizobacteria-mediated ISR resembles classical pathogen-induced systemic acquired resistance (SAR), in which noninfected parts of locally infected plants become more resistant to further infection (Ross 1961). *Pseudomonas fluorescens* strain WCS417r has been shown to effectively 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 thaliana* [Pieterse et al. 1996, 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* 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; Ton et al. 2002b; Van Wees et al. 1997). The spectrum of effectiveness of WCS417r-mediated ISR and pathogen-induced SAR overlaps, but also is partly divergent. For instance, SAR is effective against *Turnip crinkle virus*, whereas ISR is not. Conversely, ISR is effective against *A. brassicicola*, whereas SAR is not (Ton et al. 2002b). In the case of *P. syringae* pv. *tomato* DC3000, which is affected by both ISR and SAR, the level of induced resistance can be enhanced further when both types of induced resistance are activated simultaneously (Van Wees et al. 2000), indicating that the effects of ISR and SAR are additive.

Although both rhizobacteria-mediated ISR and pathogen-induced SAR are each effective against a broad spectrum of pathogens, their signal-transduction pathways are clearly distinct. The onset of SAR is accompanied by a local and systemic increase in the endogenous levels of salicylic acid (SA) (Malamy et al. 1990; Métraux et al. 1990) and the concomitant up-regulation of a large set of genes, including those encoding pathogenesis-related (PR) proteins (Maleck et al. 2000; Ward et al. 1991). Several PR proteins possess antimicrobial activity and are thought to contribute to the state of resistance attained (Van Loon and Van Strien 1999). Transduction of the SA signal requires the function of the regulatory protein NPR1 (also known as NIM1) (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). Interaction of NPR1 with the b-ZIP transcription factor TGA2 is required for activation of the SA-regulated gene *PR-1*, suggesting that NPR1 acts by altering the activity of transcription factors (Fan and Dong 2002). In contrast to SAR, WCS417r-mediated ISR functions independently of SA. This was demonstrated by the observation that *Arabidopsis* genotypes that are impaired in SA accumulation (i.e., NahG,

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eds5, and *sid2*) display levels of ISR equal to those of wild-type plants upon colonization of the roots by WCS417r (Pieterse et al. 1996, 2002; Ton et al. 2002a). Analysis of the jasmonic acid (JA)-response mutant *jar1-1*, a range of ethylene (ET)-response mutants, and the SAR-compromised mutant *npr1-1* revealed that components of the JA and ET response are required for triggering ISR and that this induced resistance response, like SAR, 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. 1997, 1999).

Apart from WCS417r, other fluorescent *Pseudomonas* spp. strains also have been shown to induce the SA-independent ISR pathway in *Arabidopsis* spp. (Iavicoli et al. 2003; Ryu et al. 2003; Van Wees et al. 1997), 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. However, not all resistance-inducing rhizobacteria trigger an SA-independent resistance. For instance, an SA-overproducing mutant of *Pseudomonas aeruginosa* 7NSK2 and the genetically modified, SA-overproducing *P. fluorescens* strain P3 have been shown to trigger the SA-dependent SAR pathway by producing SA at the root surface (De Meyer and Höfte 1997; Maurhofer et al. 1998).

In *Arabidopsis* spp., both JA and ET activate specific sets of defense-related genes (Schenk et al. 2000) and, when applied exogenously, both can induce resistance (Pieterse et al. 1998; Van Wees et al. 1999). To investigate whether ISR is associated with changes in JA- or ET-responsive gene expression, Van Wees and associates (1999) monitored the expression of a set of well-characterized JA- or ET-responsive genes (i.e., *LOX1*, *LOX2*, *VSP, PDF1.2, HEL*, *CHI-B*, and *PAL1*) in *Arabidopsis* plants expressing WCS417r-mediated ISR. None of the genes tested was up-regulated in induced plants, neither locally in the roots nor systemically in the leaves. This suggests 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). Therefore, it was assumed that the JA and ET dependency of ISR is based on enhanced sensitivity to these hormones, rather than on an increase in their production.

The onset of SAR is accompanied by substantial transcriptional reprogramming (Maleck et al. 2000), resulting in the accumulation of *PR*-gene products to levels from 0.3 to 1% of the total mRNA and protein content (Lawton et al. 1995). Although resulting in a similar enhanced resistance against different types of pathogens, ISR has not been associated with changes in gene expression, but should be likewise dependent on additional defensive activity (Pieterse et al. 2002; Van Wees et al. 1999). Therefore, plants must possess as yet undiscovered defense-related genes, whose products contribute to the broad-spectrum resistance associated with ISR. To identify novel defense-related genes that potentially are involved in rhizobacteria-mediated ISR, we determined expression levels of approximately 8,000 genes, representing approximately one-third of the *Arabidopsis* genome, in ISR-expressing plants at different time points before and after challenge inoculation with the pathogen *P. syringae* pv. *tomato* DC3000. Analysis of the expression patterns revealed 97 genes that show a substantial change in the level of expression in the roots upon colonization by WCS417r. In contrast, in the leaves, none of the approximately 8,000 genes showed a consistent change in expression level, indicating that the onset of ISR in the leaves is not associated with detectable changes in gene expression. However, after pathogen challenge, a large set of pathogen-responsive genes showed a specific or augmented change in the level of expression in the ISR-expressing plants. These results indicate that ISR-expressing plants are primed to react faster to pathogen challenge.

RESULTS

Transcript profile of *Arabidopsis* roots colonized by ISR-inducing *P. fluorescens* WCS417r.

To determine the transcript profile of *Arabidopsis* roots in response to colonization by ISR-inducing WCS417r bacteria, 2-week-old seedlings of *Arabidopsis* accession Col-0 were cultivated in a rock-wool-based system. This system previously was demonstrated to be well-suited for studying ISR in *Arabidopsis* (Pieterse et al. 1996; Van Wees et al. 1997) and has the advantage that clean root material can be collected easily for RNA extraction. For induction of ISR, a suspension of WCS417r bacteria in 10 mM $MgSO_4$, mixed with talcum powder as a carrier, was applied to the roots. As a control, 10 mM $MgSO_4$ was applied in a similar manner. Systemic protection in ISR-expressing plants results from the continuous stimulation of the plant by ISR-inducing rhizobacteria on 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; data not shown). Therefore, to survey the transcript profile of roots of *Arabidopsis* plants during the onset of ISR, root samples were collected at 3 and 7 days after the start of WCS417r treatment.

RNA was prepared from two independent biological replicates, each consisting of approximately 75 root systems. These two replicates were pooled to reduce noise arising from variation in experimental conditions. The transcript profile of each pool was obtained by hybridization of an Affymetrix *Arabidopsis* GeneChip microarray representing approximately 8,000 *Arabidopsis* genes (Zhu and Wang 2000). On this microarray, each gene is represented by at least one "probe set" consisting of 16 to 20 25-mer oligonucleotides. After hybridization, expression values from each pooled sample were normalized globally. To validate the global normalization, the fold change in expression level of a set of 10 genes previously identified as representative, constitutively expressed controls (Kreps et al. 2002), was calculated. As expected, the fold change ratio in WCS417r- over mock-treated roots was close to 1 for most of these genes (Table 1).

To identify WCS417r-responsive genes, the following conservative selection criteria were applied. First, the expression level had to be >40 in at least one of the data sets. Second, the change in expression level in WCS417r-treated roots compared with that in mock-treated roots had to be at least two-fold. Under these conditions, the technical false positive rate is approximately 0.25%, representing approximately 20 genes among the 8,000 genes analyzed by the GeneChip (Zhu and Wang 2000). A total of 1,691 probe sets met these two conditions on at least one time point after treatment of the roots with WCS417r. However, to avoid false positives, we required the changes to occur at both time points in the same direction. Therefore, only those probe sets were selected that met these selection criteria at both time points tested. Finally, 102 probe sets representing 97 genes met these stringent selection conditions, the majority of which were down-regulated (62%) (Table 2). Of the 97 genes that showed consistent changes, 18% are annotated as "unclassified proteins", 18% are predicted to be involved in cell rescue and defense, 14% in metabolism, 15% in regulating gene transcription, and 7% in cellular communication and signal transduction. In view of the ET dependency of ISR, genes encoding a putative ACC oxidase, ET response

factor 1 (ERF1), and ET responsive element binding factors 1 (EREBP1) and 2 (EREBP2) are of particular interest. Among all of the WCS417r-responsive genes, the transcript for a putative flavonol reductase was observed as the most strongly induced (i.e., over 14-fold).

To verify the GeneChip results, we selected two up-regulated genes encoding an MYB-like transcription factor (MYB72; probe set 12725_r_at) and an unknown "expressed protein" (probe set 18721_at), a down-regulated gene encoding an MLO-like protein (MLO8; probe set 13687_s_at), a gene with unchanged expression encoding vegetative storage protein 2 (VSP2; probe set 14675_s_at), and a gene encoding a glucosyltransferase, of which the transcript levels were below the detection level of the GeneChip (probe set 17362_at), and analyzed their transcript levels in WCS417r- and mock-treated *Arabidopsis* roots by reverse-transcriptase polymerase chain reaction (RT-PCR). To this end, in an independent experiment, RNA was isolated from roots of 3-week-old Col-0 plants that were grown for 7 days in the presence or absence of WCS417r bacteria in the rockwool system. The transcript levels of the selected up-regulated MYB72 transcription factor gene and the unknown "expressed protein" gene clearly were increased in WCS417r-treated roots (Fig. 1), whereas the mRNA level of the selected down-regulated *MLO8* gene clearly was lower in WCS417r-treated roots. As expected, transcript levels of the selected *VSP2* gene remained unchanged in the roots, whereas the transcript levels of the selected glucosyltransferase gene also were undetectable by RT-PCR. These results confirm the data from the GeneChip experiments.

ISR is not associated with systemic changes in gene expression in the absence of pathogen infection.

Colonization of the roots of *Arabidopsis* accession Col-0 by WCS417r results in a systemic resistance in the leaves that is effective against different types of pathogens (Pieterse et al. 2002). To identify genes that show a specific change in expression in the leaves in response to treatment of the roots with ISR-inducing rhizobacteria, 2-week-old Col-0 seedlings were transplanted into soil with or without WCS417r. To examine the expression profile of leaves of induced and noninduced plants, leaf samples were collected 3 and 7 days after induction. Expression of ISR routinely was verified in parallel using our standard bioassay (Pieterse et al. 1996) with the bacterial leaf pathogen *P. syringae* pv. *tomato* DC3000 as the challenging pathogen (data not shown). RNA was prepared from three independent biological replicates, each consisting of approximately 25 rosettes, that were pooled to reduce noise arising from variation in experimental conditions. Transcript profiling was performed for two independent experiments on data sets that were normalized globally. Global normalization was validated again by calculating the fold change in expression level of the 10 representative constitutively expressed control genes. The fold change ratio in leaves of WCS417r- over mock-treated plants was close to 1 for most of these genes (Table 3).

To identify genes that respond systemically in the leaves to colonization of the roots by WCS417r, we selected probe sets that had an expression level of >40 and that showed a greater than twofold change in ISR-expressing leaves compared with noninduced leaves. The number of probe sets that met these criteria on the single time points varied between 20 and 23, which is close to the technical false positive rate of approximately 0.25% (Zhu and Wang 2000). To reduce false positives, we required the changes to be consistent at both time points tested or to show reproducibility between experiments. Interestingly, none of the probe sets met the selection criteria. Northern blot analysis of the RNA samples that were used for the GeneChip hybridizations and of RNA samples from similar other experiments confirmed that the transcript levels of several defense-related genes (e.g., *CHI-B, HEL*, *LOX2*, *PDF1.2*, *PR-1*, *PR-2*, and *PR-5*) were not increased in the leaves in response to colonization of the roots by ISR-inducing rhizobacteria (Van Wees et al. 1999; data not shown). These results indicate that, although ISR-expressing leaves possess an enhanced defensive capacity, the state of ISR is not associated with detectable changes in the expression of the approximately 8,000 genes represented on the GeneChip.

Selection of *P. syringae* pv. *tomato* DC3000-responsive genes in control and ISR-expressing plants.

The observed lack of changes in gene expression in leaves of WCS417r-induced plants suggests that the broad-spectrum effectiveness of ISR might be based on processes that are apparent only after pathogen attack. Previously, Northern blot analyses of the JA-responsive, pathogen-inducible gene *VSP2* revealed that ISR is associated with enhanced expression of this gene after infection of the leaves by *P. syringae* pv. *tomato* DC3000 (Van Wees et al. 1999), suggesting that ISR-expressing plants are primed to express specific pathogen-inducible genes at a higher level after challenge. To investigate the transcript profile of pathogen-responsive genes in ISR-expressing plants, *Arabidopsis* Col-0 plants grown in soil with or without ISR-inducing WCS417r bacteria were challenge inoculated with *P. syringae* pv. *tomato* DC3000 and checked for expression of ISR (data not shown). Leaf samples were collected from control and WCS417r-induced plants at 0, 6, and 24 h after challenge inoculation. RNA was prepared from three

Table 1. Fold-change ratio of representative constitutively expressed control genes in *Pseudomonas fluorescens* WCS417r-treated compared with mock-treated *Arabidopsis* roots

Annotation	Fold-change ratio[a]		Probe set no.	AGI no.
	3 days	7 days		
Polyubiquitin, UBQ10	0.66	0.57	12833_f_at	AT4G05320
Eukcaryotic initiation factor elF-4A1	1.66	0.98	16026_at	AT3G13920
Aquaporin, PIP-1B	2.74	1.15	15977_s_at	AT2G45960
V-type H^+-ATPase, 16 kD-subunit	2.00	0.99	15584_s_at	AT1G19910
40S ribosomal protein S16	1.17	1.05	17390_at	AT2G09990
Actin 2	1.71	1.21	16476_at	AT3G18780
Plasma membrane H^+-ATPase, AHA1	1.38	1.08	14713_s_at	AT2G18960
Tubulin, ß-4	0.72	1.33	15988_at	AT5G44340
Calmodulin-1	1.52	1.03	15173_f_at	AT5G37780
Ca-dependent protein kinase, CPK3	1.57	1.15	17058_s_at	AT4G23650
Average	1.5	1.1	…	…

[a] Fold change ratios (WCS417r/mock) are based on gene expression profiles of roots of Col-0 plants at 3 or 7 days after treatment with induced systemic resistance-inducing WCS417r bacteria or 10 mM $MgSO_4$ (mock).

independent biological replicates, each consisting of approximately seven rosettes, that were pooled to reduce noise arising from variation in experimental conditions. To identify *P. syringae* pv. *tomato* DC3000-responsive genes in noninduced and in ISR-expressing plants, probe sets were selected that showed an expression level of >40 and a greater than twofold change at 6 or 24 h after pathogen inoculation compared with nonchallenged plants. A total of 1,661 probe sets satisfied this condition in noninduced plants, whereas 1,507 probe sets met the selection criteria in WCS417r-induced plants. The lower number of selected probe sets from the data set of WCS417r-induced plants correlated with the observed reduction of disease symptoms in the ISR-expressing plants (data not shown). Only those probe sets were selected that met the above-mentioned selection criteria at both 6 and 24 h after challenge inoculation, resulting in a total of 523 probe sets representing 469 genes in noninduced plants, and 479 probe sets representing 425 genes in WCS417-induced plants.

Table 2. Fold-change ratio of *Pseudomonas fluorescens* WCS417r-responsive genes in WCS417r-treated compared with mock-treated *Arabidopsis* roots

Annotation[b]	Fold-change ratio[a]		Probe set no.	AGI no.
	3 days	7 days		
Transcription				
Myb family transcription factor	7.4	3.4	18479_at	AT3G12820
Identical to WRKY transcription factor 31	3.0	2.4	18213_at	AT4G22070
Myb family transcription factor (MYB72)	2.8	3.1	12725_r_at	AT1G56160
GATA zinc finger protein	2.3	3.7	13168_i_at	AT2G45050
Putative chloroplast nucleoid DNA binding protein	2.2	2.1	15720_at	AT2G03200
Myb family transcription factor (MYB88)	–2.5	–2.9	14852_s_at	AT2G02820
Putative C2H2-type zinc finger protein	–2.5	–2.1	20620_g_at	AT2G37430
CONSTANS B-box zinc finger family protein	–2.7	–2.1	19855_at	AT1G78600
No apical meristem (NAM) protein family	–2.8	–2.0	18590_at	AT1G69490
Myb family transcription factor	–3.4	–2.5	19707_s_at	AT5G67300
RING-H2 finger protein RHA1a -like protein	–3.7	–2.2	16130_s_at	AT4G11370
Ethylene response factor 1	–6.0	–2.2	17514_s_at	AT3G23240
Putative MYB family transcription factor (MYB25)	–6.3	–2.4	17606_s_at	AT2G39880
Ethylene responsive element binding factor 2	–8.7	–2.3	16609_at	AT5G47220
Ethylene responsive element binding factor 2	–24.9	–3.3	12905_s_at	AT5G47220
Ethylene responsive element binding factor 1	–59.1	–2.6	12904_s_at	AT4G17500
Cell rescue and defense				
Peroxidase	4.1	3.9	19622_g_at	AT5G42180
Putative protein	3.5	2.3	13973_at	AT4G36980
Peroxidase, putative	3.3	3.1	12386_at	AT1G44970
RAS-related GTP-binding protein (ARA-1)	3.3	2.1	18195_at	AT1G05810
Drought-induced protein like (Di21)	2.2	2.1	18231_at	AT4G15910
Polyubiquitin (UBQ4)	–2.4	–2.7	12830_f_at	AT5G20620
Peroxidase ATP5a	–2.4	–2.5	19602_at	AT1G49570
Expressed protein	–2.6	–3.1	15083_at	AT4G32190
Expressed protein	–2.8	–2.0	12114_at	AT4G39680
Pathogenesis-related protein 1 precursor	–2.9	–3.1	18451_s_at	AT4G33710
Cytochrome P450	–3.0	–2.0	14248_at	AT3G26830
Polyubiquitin (UBQ4)	–3.1	–3.0	12831_f_at	AT5G20620
Peroxidase, putative	–3.6	–2.6	12475_at	AT5G06730
Zinc finger protein 5, ZFP5	–4.0	–2.8	16582_s_at	AT1G10480
Seven transmembrane MLO protein family (MLO8)	–4.8	–3.3	13687_s_at	AT2G17480
Pathogenesis-related protein 1 precursor	–5.1	–2.5	20308_s_at	AT4G33720
Heavy-metal-associated domain-containing protein	–8.3	–2.4	14485_at	AT1G01490
Germin-like protein (GLP10)	–8.7	–4.5	17037_s_at	AT3G62020
Systemic regulation of or/ interaction with cellular environment				
Similar to gibberellin-regulated proteins	3.9	2.9	19863_at	AT2G14900
GAST1 protein homolog	3.6	2.6	15121_s_at	AT1G75750
GAST1 protein homolog	3.5	2.7	16014_s_at	AT1G75750
Putative amino-cyclopropane-carboxylic acid oxidase	2.5	2.0	18310_at	AT1G12010
Gibberellin 3 beta-hydroxylase, putative (GA4)	–2.9	–8.2	17549_at	AT1G15550
LAX1/AUX1–like permease	–3.7	–2.1	18667_at	AT5G01240
Terpene synthase/cyclase family	–5.7	–2.2	17511_s_at	AT1G61120
Cellular communication or signal transduction				
Leucine rich repeat family	9.0	4.0	16408_at	AT4G18760
Expressed protein	2.6	2.1	12154_at	AT2G35190
Expressed protein	–2.6	–2.4	14096_at	AT1G76960
CBL-interacting protein kinase 4	–3.5	–2.0	12395_s_at	AT4G14580
Protein kinase (ADK1)	–3.9	–3.0	15692_s_at	AT1G03930
Putative protein/phospholipase C	–6.1	–2.0	12213_at	AT4G34920
Putative protein/leucine-rich-repeat protein	–9.0	–2.3	15249_at	AT4G29880
Metabolism				
Dihydroflavonol 4-reductase (dihydrokaempferol 4-reductase) family	14.4	14.3	18198_at	AT2G45400
Terpene synthase/cyclase family	9.9	2.1	18127_at	AT4G20230
1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR)	8.8	2.5	12218_at	AT5G62790
Short chain dehydrogenase/reductase family protein (b-keto acyl reductase, putative)	3.0	2.2	17378_at	AT1G67730

(*continued on next page*)

[a] Fold-change ratios (WCS417r/mock) are based on gene expression profiles of roots of Col-0 plants, 3 and 7 days after treatment with 10 mM $MgSO_4$ (mock) or induced systemic resistance-inducing WCS417r bacteria.

[b] Annotations are as predicted by the MIPS *Arabidopsis thaliana* Genome Database.

ISR-expressing plants are primed for augmented pathogen-responsive gene expression.

Having identified *P. syringae* pv. *tomato* DC3000-responsive genes, we tested our hypothesis that ISR-expressing plants are primed to respond faster or with a greater magnitude to pathogen infection. To this end, we compared the expression levels of the *P. syringae* pv. *tomato* DC3000-responsive genes in noninduced and ISR-expressing plants. To identify *P. syringae* pv. *tomato* DC3000-responsive genes that show an augmented expression pattern in ISR-expressing plants (so-called ISR-primed genes), we required the change in expression level of the *P. syringae* pv. *tomato* DC3000-responsive genes to be >1.5-fold higher in the WCS417r-treated plants. This latter criterion was based on quantitative expression data of the *VSP2* gene, which previously was demonstrated to be primed in ISR-expressing plants (Van Wees et al. 1999). In several independent experiments, *VSP2* transcripts consistently accumulated to an approximately 1.5-

Table 2. (*continued from preceding page*)

Annotation	Fold-change ratio 3 days	Fold-change ratio 7 days	Probe set no.	AGI no.
Metabolism (*continued*)				
FAD-linked oxidoreductase family	2.3	2.4	13622_i_at	AT4G20820
Glycosyl hydrolase family 1	–2.2	–2.1	16778_at	AT2G44480
Glycine-rich RNA binding protein (AtGRP7)	–2.5	–2.6	15105_s_at	AT2G21660
Putative tyrosine aminotransferase	–3.0	–2.3	17008_at	AT2G24850
Copper amine oxidase-like protein	–3.9	–2.0	20555_s_at	AT4G12280
Glycosyltransferase family 20	–4.7	–2.4	13706_s_at	AT2G18700
Geranylgeranyl pyrophosphate synthase (ggps6)	–5.8	–4.6	13257_s_at	AT1G49530
S-adenosylmethionine decarboxylase	–6.7	–2.3	16437_s_at	AT3G02470
Lipase (class 3) family	–6.9	–2.6	14358_s_at	AT4G16820
Ferrochelatase-I	–8.3	–3.0	12571_s_at	AT5G26030
Protein synthesis				
Translation initiation fact. eIF-2 gamma subunit, putative	–11.1	–3.0	17941_at	AT2G18720
Putative translation initiation factor eIF-2B delta subunit	–29.5	–2.5	16255_at	AT2G44070
Transport facilitation				
Unknown protein/cation transport protein	3.0	3.2	15544_at	AT4G31290
Zinc transporter (ZIP2)	2.3	2.0	15666_s_at	AT5G59520
Monooxygenase family	–3.0	–2.5	17051_s_at	AT2G29720
Glucose-6-phosphate/phosphate-translocator precursor, putative	–9.6	–2.1	17775_at	AT1G61800
DNA damage-inducible protein (EDS5/SID1)	–14.0	–3.5	17653_at	AT4G39030
Cell cycle and DNA processing				
Putative AAA-type ATPase	2.1	2.0	16345_at	AT2G03670
Unknown protein	–3.3	–2.3	14130_at	AT1G03080
Development (systemic)				
Nodulin-like protein (mtn21)	2.5	7.6	16258_at	AT2G39510
Control of cellular organization				
Actin depolymerizing factor-like protein	7.1	9.8	19684_at	AT4G34970
Small heat shock protein	2.1	2.5	13282_s_at	AT4G25200
Energy				
Expressed protein	2.9	2.1	15851_i_at	AT2G27370
Nitrate reductase 2 (NR2)	–5.8	–3.8	14242_s_at	AT1G37130
Nitrate reductase 1 (NR1)	–14.1	–11.4	14240_s_at	AT1G77760
Nitrate reductase 1 (NR1)	–41.7	–9.0	18899_s_at	AT1G77760
Subcellular localization				
Unknown protein	–6.3	–3.2	14524_s_at	AT1G65580
Cell fate				
Expressed protein	9.5	9.8	18721_at	AT3G02040
Expressed protein	–2.5	–2.8	18346_at	AT4G35890
Protein fate (folding, modification, destination)				
DegP protease	–2.1	–2.3	16067_at	AT3G27925
Putative DnaJ protein	–11.7	–6.2	15367_at	AT1G76700
Serine carboxypeptidase -related	–27.8	–3.4	18132_at	AT4G15100
Unclassified proteins				
Lateral organ boundaries (LOB) domain family	9.2	2.4	15808_at	AT2G30130
Auxin-induced protein-related	4.3	4.1	15017_at	AT2G24400
Auxin-induced protein-related	4.2	3.2	16751_at	AT4G34750
Putative OBP32pep protein	3.5	9.7	13855_at	AT1G23590
Putative protein	3.5	3.0	20487_at	AT4G34810
Glucose-methanol-choline (GMC) oxidoreductase family	2.7	3.8	19068_i_at	AT1G14185
Expressed proteins	2.5	3.1	14436_at	AT3G50750
Unknown protein	2.3	2.1	15861_at	AT2G35850
Expressed protein	2.3	2.2	15918_at	AT1G30750
Expressed protein	2.1	2.4	12163_at	AT2G42780
Putative protein	–2.0	–9.5	13963_at	AT4G18890
RWP-RK domain containing protein	–2.1	–2.5	14521_at	AT4G38340
Kelch repeat containing F-box protein family	–2.2	–2.1	12695_at	AT4G38940
Cytochrome p450 family	–2.7	–3.0	18951_at	AT4G15330
Expressed protein	–3.9	–2.6	15933_at	AT1G21830
Expressed protein	–5.0	–2.6	18160_at	AT4G16745
Putative protein	–5.3	–2.0	20179_at	AT4G38060
Putative tropinone reductase	–73.1	–2.6	20370_at	AT2G29150

fold higher level in *P. syringae* pv. *tomato* DC3000-inoculated, ISR-expressing plants than in similarly inoculated control plants (data not shown). Therefore, to select for ISR-primed genes, we required their expression levels to be at least 1.5-fold higher in WCS417r-treated plants. Note that, in addition to this selection criterion, the *P. syringae* pv. *tomato* DC3000-responsive genes already were preselected to show an expression level of >40 and a greater than twofold change at both 6 and 24 h after infection.

A comparison of the changes in transcript levels of the ISR-primed, *P. syringae* pv. *tomato* DC3000-responsive genes in control and ISR-expressing Col-0 plants is depicted in Figure 2. A total of 52 probe sets representing 51 genes met the selection conditions (Table 4). The previously identified ISR-primed, *P. syringae* pv. *tomato* DC3000-responsive gene *VSP2* was among those in this group, illustrating the validity of this analysis. Other ISR-primed genes of particular interest are the JA- and ET-responsive gene *PDF1.2*, a thaumatin-like gene, a chitinase gene, and a gene encoding EREBP2. In addition to the 51 selected ISR-primed genes, 30 genes showed a *P. syringae* pv. *tomato* DC3000-induced change in WCS417r-treated plants only; in noninduced plants, the mRNA levels of the corresponding genes remained unchanged after pathogen infection (Fig. 2; Table 5). Apparently, these genes respond to *P. syringae* pv. *tomato* DC3000 infection only in ISR-induced plants. This latter group is further referred to as ISR-specific. Of the 30 ISR-specific genes, 6 are annotated as "unclassified proteins", 5 are predicted to be involved in metabolism, and 4 genes are likely to be involved in regulating gene transcription (Table 5). Moreover, five genes are predicted to be involved in signal transduction. One of those shows homology to *ERF1*, which encodes a transcription factor that acts downstream of ET and JA signaling and has been implicated in the defense response of *Arabidopsis* against various pathogens (Berrocal-Lobo et al. 2002; Lorenzo et al. 2003; Solano et al. 1998).

To verify the GeneChip data, we examined the expression levels of the *P. syringae* pv. *tomato* DC3000-responsive gene *VSP1* (probe set 15125_f_at), the ISR-primed *P. syringae* pv. *tomato* DC3000-responsive genes *PDF1.2* (probe set 14621_at) and *VSP2* (probe set 14675_s_at), and the constitutively expressed ubiquitin gene *UBQ10* (probe set 12835_f_at) in an independent experiment. To this end, Northern blot analysis was performed using RNA from leaves of 5-week-old control and ISR-expressing plants that were harvested 0 and 24 h after inoculation with *P. syringae* pv. *tomato* DC3000. Transcript levels of *VSP1, VSP2,* and *PDF1.2* clearly were increased in response to *P. syringae* pv. *tomato* DC3000 infection (Fig. 3), whereas the expression of *UBQ10* remained unchanged. Moreover, *VSP2* and *PDF1.2* showed a clearly enhanced level of expression in challenged ISR-expressing plants over challenged control plants, whereas the *P. syringae* pv. *tomato* DC3000-induced expression level of *VSP1* remained unchanged. The level of enhanced expression of *VSP2* and *PDF1.2* in challenged ISR-expressing plants over *P. syringae* pv. *tomato* DC3000-inoculated control plants was similar to that observed in the Gene-

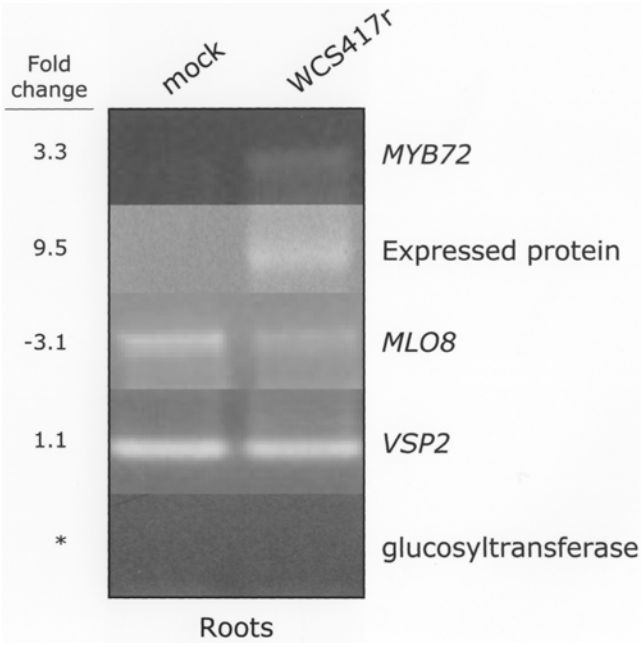

Fig. 1. Verification of root GeneChip data. Transcript levels of genes that were selected in the GeneChip analysis as being up-regulated, down-regulated, unchanged (see fold change), or undetectable (asterisk) in WCS417r-treated roots were analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR). Shown are ethidium bromide-stained agarose gels with RT-PCR products obtained after amplification of equal portions of first-strand cDNA using gene-specific primers of the genes indicated (corresponding AGI numbers are AT1G56160, AT3G02040, AT2G17480, AT5G24770, and AT4G15260). First-strand cDNA was synthesized on mRNA that was isolated from roots of *Arabidopsis* Col-0 plants, 7 days after treatment of the roots with 10 mM $MgSO_4$ (mock) or induced systemic resistance-inducing WCS417r bacteria.

Table 3. Fold-change ratio of representative constitutively expressed control genes in leaves of *Pseudomonas fluorescens* WCS417r-treated compared with mock-treated *Arabidopsis* plants, before and after challenge inoculation with *P. syringae* pv. *tomato* DC3000

	Fold-change ratio				
	Before challenge[a]		After challenge[b]		
Annotation	3 days	7 days	0 h	6 h	24 h
Polyubiquitin, UBQ10	0.79	1.08	1.05	1.39	0.85
Eukcaryotic initiation factor elF-4A1	1.01	1.20	1.08	1.02	0.88
Aquaporin, PIP-1B	1.03	1.12	0.94	0.98	0.94
V-type H^+-ATPase, 16 kD-subunit	0.97	0.99	0.98	1.05	1.01
40S ribosomal protein S16	1.07	1.03	1.15	1.02	1.01
Actin 2	0.98	1.02	1.14	1.29	1.27
Plasma membrane H^+-ATPase, AHA1	1.43	0.79	0.96	0.99	1.23
Tubulin, β-4	1.24	1.35	1.04	0.98	0.87
Calmodulin-1	1.04	1.13	0.93	0.92	1.03
Ca-dependent protein kinase, CPK3	1.08	0.64	1.04	1.06	1.01
Average	1.1	1.0	1.0	1.1	1.0

[a] Fold-change ratios (WCS417r/mock) are based on gene expression profiles of leaves of Col-0 plants at 3 and 7 days after treatment of the roots with induced systemic resistance-inducing WCS417r bacteria or 10 mM $MgSO_4$ (mock).

[b] Fold-change ratios (WCS417r/mock) are based on gene expression profiles of leaves of Col-0 plants, grown in the presence or absence of WCS417r bacteria, at 0, 6, and 24 h after challenge inoculation with *P. syringae* pv. *tomato* DC3000.

Chip data analysis ("ratio" in Table 4). These results agree with and confirm the corresponding data of the GeneChip data analysis.

The role of JA and ET in priming.

Arabidopsis mutants affected in their ability to respond to either JA or ET are compromised in WCS417r-mediated ISR, indicating that the expression of ISR requires an intact JA and ET signaling pathway (Pieterse et al. 1998, 2002). To investigate the possible role of JA and ET in the regulation of ISR-primed genes, we made use of a microarray data set previously published by Glazebrook and associates (2003). This data set consists of global expression patterns of *P. syringae* pv. *maculicola* ES4326-induced genes in wild-type *Arabidopsis* Col-0 and various signaling-defective mutant plants. Previously, Tao and associates (2003) demonstrated that the expression profile of wild-type Col-0 plants inoculated with either *P. syringae* pv. *tomato* DC3000 or *P. syringae* pv. *maculicola* ES4326 is very similar. Therefore, we assumed that the global expression phenotype of *P. syringae* pv. *maculicola* ES4326-responsive genes is very similar to that of *P. syringae* pv. *tomato* DC3000-responsive genes. To validate this assumption, we compared the group of *P. syringae* pv. *tomato* DC3000-responsive genes in control plants with the group of *P. syringae* pv. *maculicola* ES4326-responsive genes that showed an expression level of >40 and a greater than twofold change compared with mock-inoculated plants. Of the 523 *P. syringae* pv. *tomato* DC3000-responsive probe sets that showed a consistent change in expression in noninduced *P. syringae* pv. *tomato* DC3000-infected Col-0 plants, a large group of 278 probe sets (53%) showed a similar expression pattern in *P. syringae* pv. *maculicola* ES4326-infected Col-0 plants. This group of *P. syringae* pv. *tomato*- and *P. syringae* pv. *maculicola*-responsive genes was used in the analysis of the data sets that were obtained with the signaling-defective genotypes (see below). The expression level of the remaining *P. syringae* pv. *tomato* DC3000-responsive genes did not appear to change substantially in *P. syringae* pv. *maculicola* ES4326-inoculated plants. This might be due to differences in experimental set-up, because the expression profile of the *P. syringae* pv. *maculicola* ES4326-responsive genes was determined at 30 h after pressure infiltrating of *P. syringae* pv. *maculicola* ES4326 into the leaves, whereas our data were collected at 6 and 24 hours after dipping the leaves in a suspension of *P. syringae* pv. *tomato* DC3000.

To determine the involvement of JA, ET, and SA in the regulation of the ISR-primed, *P. syringae* pv. *tomato* DC3000-responsive genes, we compared their expression profile in *P. syringae* pv. *maculicola* ES4326-infected wild-type Col-0 plants and the various signaling-defective transgenic or mutant genotypes in the Col-0 background. These genotypes were *coi1-1*, which blocks JA signaling (Feys et al. 1994; Xie et al. 1998); *ein2-1*, which blocks ET signaling (Alonso et al. 1999; Guzmán and Ecker 1990); and *eds5-1* and NahG, which are affected in SA signaling (Gaffney et al. 1993; Nawrath et al. 2002). Of the 51 ISR-primed *P. syringae* pv. *tomato* DC3000-responsive genes, 29 genes (57%) also were responsive to *P. syringae* pv. *maculicola* ES4326. This set of ISR-primed, *P. syringae* pv. *tomato*- and *P. syringae* pv. *maculicola*-responsive genes was further analyzed in the signaling-defective genotypes.

To analyze the role of JA, ET, and SA in the regulation of the *P. syringae* pv. *tomato*- and *P. syringae* pv. *maculicola*-responsive genes, we compared their expression levels in wild-type Col-0 plants with those in the various signaling-defective genotypes. A gene was scored as JA-, ET-, or SA-responsive when an altered *P. syringae* pv. *maculicola* ES4326 response was observed in the respective mutant or transgenic compared with the wild type, or when the change in expression differed at least 1.5-fold compared with that observed in *P. syringae* pv. *maculicola* ES4326-infected Col-0 plants. Of all the 278 *P. syringae* pv. *tomato*- and *P. syringae* pv. *maculicola*-responsive probe sets, 12% were dependent on SA signaling only; 30% were regulated by JA signaling, ET signaling, or both; and 22% were affected by a combination of JA, ET, and SA signaling (Fig. 4, top panel). The remaining 36% of the *P. syringae* pv. *tomato*- and *P. syringae* pv. *maculicola*-responsive genes showed a similar expression pattern in all genotypes tested, indicating that their expression was not affected by either of these signals. Analysis of the ISR-primed, *P. syringae* pv. *tomato*- and *P. syringae* pv. *maculicola*-responsive genes revealed that only 3% were dependent solely on SA signaling, 35% of the genes were regulated by JA and ET signaling, and

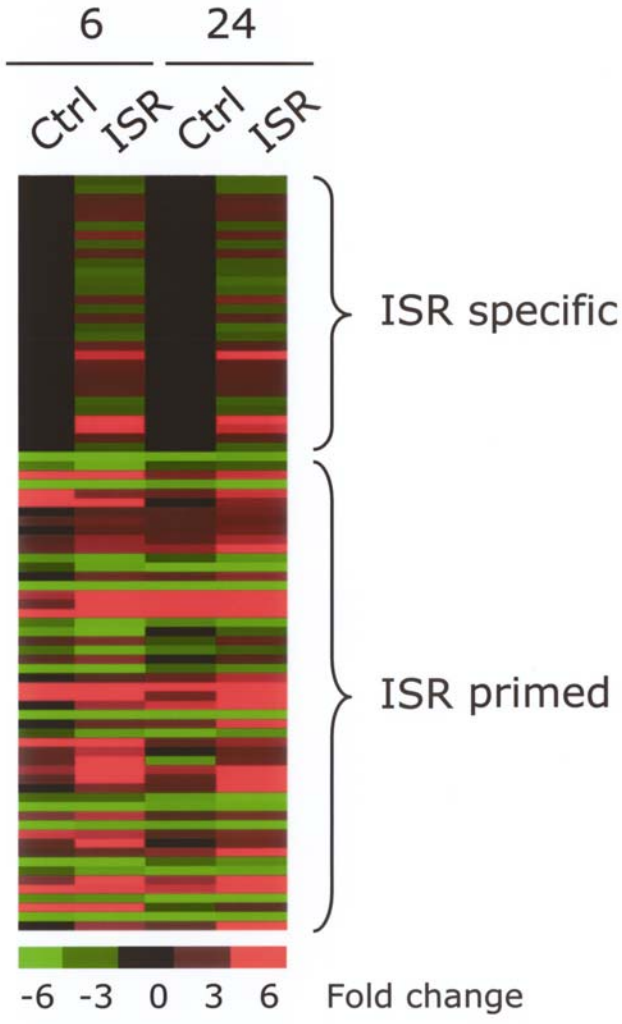

Fig. 2. Augmented expression of *Pseudomonas syringae* pv. *tomato* DC3000-responsive genes in *Arabidopsis* plants expressing *P. fluorescens* WCS417r-mediated induced systemic resistance (ISR). Comparison of the changes in transcript levels of ISR-specific and ISR-primed genes in control and ISR-expressing Col-0 plants at 6 and 24 h after inoculation with *P. syringae* pv. *tomato* DC3000. ISR-specific genes show a consistent >twofold change in the level of expression at 6 and 24 h after inoculation, whereas these genes remained unchanged in *P. syringae* pv. *tomato* DC3000-inoculated control plants. ISR-primed genes display a consistent >twofold change in both control and ISR-expressing plants at 6 and 24 h after inoculation. In addition, the magnitude of this change is >1.5-fold stronger in challenged ISR-expressing plants compared with *P. syringae* pv. *tomato* DC3000-inoculated control plants. The values are visualized by TreeView software.

Table 4. Fold-change ratios of induced systemic resistance (ISR)-primed *Pseudomonas syringae* pv. *tomato* DC3000-responsive genes in leaves of *P. fluorescens* WCS417r-treated plants[a]

	Challenged leaves, fold-change ratio							
	6 h			24 h				
Annotation[b]	**Ctrl**	**ISR**	**Ratio**	**Ctrl**	**ISR**	**Ratio**	**Probe set no.**	**AGI no.**
Transcription								
Ethylene responsive element binding factor 2	12.2	19.8	1.6	1.0	3.8	3.8	12905_s_at	AT5G47220
WRKY family transcription factor	2.0	3.1	1.5	2.2	2.9	1.3	13115_at	AT1G62300
Putative YABBY3 axial regulator	–2.2	–4.1	–1.9	–5.5	–3.8	1.4	17530_at	AT4G00180
Putative heat shock transcription factor	–11.8	–23.9	–2.0	–4.1	–6.6	–1.6	12431_at	AT2G26150
Cell rescue and defense								
Cytochrome P450 family	4.9	14.2	2.9	9.8	28.0	2.8	14609_at	AT2G30770
Thaumatin-like protein	6.3	17.0	2.7	–2.1	2.2	4.6	20384_at	AT4G36010
Antifungal protein PDF1.2	3.2	7.9	2.5	13.4	31.9	2.4	14621_at	AT5G44420
Glycosyl hydrolase fam. 19 (chitinase)	2.2	4.3	2.0	2.5	4.9	1.9	13153_r_at	AT2G43590
Glycosyl hydrolase fam. 19 (chitinase)	2.9	4.5	1.6	4.4	7.0	1.6	13154_s_at	AT2G43590
Expressed protein	–22.2	–25.2	–1.1	–13.9	–25.2	–1.8	16637_s_at	AT4G14690
Gamma-glutamyltransferase-related	–3.8	–4.5	–1.2	–2.2	–3.9	–1.8	13255_i_at	AT4G39640
Heat shock factor protein 7 (HSF7)	–6.7	–10.4	–1.5	–2.8	–3.7	–1.3	19629_at	AT4G11660
Cellular communication or signal transduction mechanism								
Receptor-related serine/threonine protein kinase ARK3	1.8	4.7	2.5	5.7	12.7	2.2	16360_at	AT4G21380
Protein kinase-like protein	3.7	8.4	2.3	4.4	12.3	2.8	20232_s_at	AT4G23130
PP1/PP2A phosphatases pleiotropic regulator PRL2	3.0	5.2	1.7	1.2	2.9	2.5	17954_s_at	AT3G16650
Ras-related GTP-binding protein (Rab7)	–3.6	–3.7	–1.0	–8.7	–14.2	–1.6	20330_at	AT1G22740
Metabolism								
Short-chain alcohol dehydrogenase-like protein	1.8	4.2	2.3	3.0	6.4	2.1	20685_at	AT4G13180
2-oxoglutarate-dependent dioxygenase (AOP2)	2.4	3.9	1.6	2.0	2.1	1.1	15238_at	AT4G03060
Putative cytochrome P450	2.1	3.0	1.5	4.0	6.5	1.6	19549_s_at	AT2G22330
Anthranilate synthase component I-1 precursor	6.4	6.0	–1.1	5.4	8.3	1.5	20291_at	AT5G05730
Arabinogalactan-protein (AGP4)	–2.9	–4.8	–1.6	–3.2	–2.4	1.3	15107_s_at	AT5G10430
Storage protein								
Vegetative storage protein VSP2	15.1	23.0	1.5	143.4	178.6	1.2	14675_s_at	AT5G24770
Putative protein/storage protein	18.0	25.7	1.4	10.6	18.7	1.8	15886_at	AT4G24350
Protein activity regulation								
Serpin, putative	2.7	6.4	2.4	-1.3	3.2	4.0	19322_at	AT1G47710
Energy								
Phosphoadenylyl-sulfate reductase (thioredoxin) (PAPS reductase)	–7.5	–13.7	–1.8	–4.4	–5.3	–1.2	18696_s_at	AT1G62180
Expressed protein	–3.0	–5.4	–1.8	–3.6	–4.3	–1.2	14917_at	AT2G35760
Glutaredoxin protein family	–2.0	–4.4	–2.1	–17.6	–17.4	1.0	13258_s_at	AT2G47880
Development (systemic)								
Aldose 1-epimerase family	1.8	3.2	1.8	2.7	4.1	1.5	13880_s_at	AT4G25900
No apical meristem (NAM) protein family	4.5	6.5	1.4	4.9	8.7	1.8	18591_at	AT5G08790
Protein with binding function or cofactor requirement								
DEAD box RNA helicase, putative	3.0	12.0	4.1	–3.9	3.1	12.2	18016_r_at	AT5G08610
DEAD/DEAH box RNA helicase protein, putative	8.9	13.8	1.6	3.4	6.5	1.9	15906_at	AT1G59990
Zinc finger protein Zat12	1.5	2.1	1.4	2.1	3.3	1.6	13015_s_at	AT5G59820
Putative zinc-finger protein	–9.6	–15.8	–1.6	–8.6	–7.9	1.1	14504_s_at	AT2G28200
Subcellular localization								
Unknown protein	–1.4	3.6	4.9	2.4	11.4	4.7	18625_at	AT1G03290
Gamma glutamyl hydrolase, putative	1.4	2.4	1.7	2.1	3.2	1.5	13118_f_at	AT1G78660
E3 ubiquitin ligase (RMA1)	6.3	9.2	1.5	2.7	4.4	1.6	17552_s_at	AT4G03510
Thioredoxin family	–2.7	–2.7	1.0	–4.2	–7.9	–1.9	18637_at	AT2G42580
Transport facilitation								
Proline transporter 2 (ProT2)	5.5	5.2	–1.1	2.3	3.5	1.5	19158_at	AT3G55740
Unclassified proteins								
Expressed protein	1.2	2.9	2.4	3.2	5.9	1.9	17010_s_at	AT2G20670
ACT domain-containing protein	2.7	6.1	2.3	2.8	7.0	2.5	18624_at	AT2G39570
Expressed protein	1.1	2.1	1.9	3.0	5.2	1.7	15479_at	AT4G26060
Expressed protein	2.2	3.6	1.6	–2.0	2.7	5.4	15063_at	AT4G12070
Protease inhibitor/seed storage/lipid transfer protein	–7.4	–4.9	1.5	–4.3	-7.3	–1.7	18983_s_at	AT4G12510
Subunit 1 of NADH dehydrogenase	3.6	5.1	1.4	2.2	3.4	1.6	18709_at	ATNADH
Expressed protein	8.0	11.6	1.4	3.6	5.6	1.6	12212_at	AT3G52070
Expressed protein	–4.0	–6.3	–1.6	–1.8	–2.4	–1.4	14923_at	AT2G28320
Expressed protein	–3.3	–5.1	–1.6	–2.1	–2.4	–1.2	12128_at	AT2G41010
Expressed protein	–2.6	–4.1	–1.6	–5.3	–4.5	1.2	19952_at	AT1G12020
Expressed protein	–16.2	–28.0	–1.7	–5.4	–4.5	1.2	12027_at	AT4G20170
Expressed protein	–5.0	–8.9	–1.8	–2.7	–3.6	–1.3	15476_at	AT2G21560
Cathepsin B-like cysteine protease, putative	7.4	3.8	–1.9	3.0	5.6	1.8	12757_at	AT1G02300
Expressed protein	–5.0	–9.9	–2.0	–7.0	–5.3	1.3	20678_at	AT1G11700

[a] Fold-change ratios (time = 6/0 h or 24/0 h) are based on transcript profiles of leaves of control (Ctrl) and ISR-expressing Col-0 plants at 0, 6, and 24 h after challenge inoculation with *P. syringae* pv. *tomato* DC3000. Numbers in "Ratio" columns express the level of augmented expression of the ISR-primed *P. syringae* pv. *tomato* DC3000-responsive genes (fold-change ratio ISR/fold-change ratio Ctrl).

[b] Annotations are as predicted by the MIPS *Arabidopsis thaliana* Genome Database.

17% of the genes were affected by a combination of JA, ET, and SA (Fig. 4, bottom panel). These results suggest that the group of ISR-primed genes is enriched for JA- and ET-responsive genes.

DISCUSSION

Rhizobacteria-mediated ISR and pathogen-induced SAR are two inducible defense responses that are controlled by distinct signaling pathways (Pieterse et al. 1998). Expression profiling of *Arabidopsis* plants expressing SAR revealed that this type of induced resistance is accompanied by substantial transcriptional reprogramming (Maleck et al. 2000), resulting in the accumulation of SAR gene products to levels from 0.3 to 1% of the total mRNA and protein content (Lawton et al. 1995). In this study, we analyzed the transcript profile of roots and leaves of *Arabidopsis* plants expressing WCS417r-mediated ISR, using a GeneChip representing approximately one-third of the *Arabidopsis* genome. A consistent shift in the expression of 102 probe sets (representing 97 genes) was observed in roots after colonization by WCS417r (Table 2), indicating that *Arabidopsis* roots respond to external stimuli perceived from this nonpathogenic rhizobacterium. These changes may be related to the roots being colonized by the bacterial strain and may be involved in the expression of localized or systemically induced resistance. The large quantity of data makes it impossible to discuss all possible functions of the identified WCS417r-responsive genes in ISR. However, in view of the ET dependency of ISR, it is worth noting that genes encoding ERF1, EREBP1, and EREBP2 are strongly down-regulated in roots that are colonized by ISR-inducing WCS417r bacteria. ERF1, EREBP1, and EREBP2 are positive regulators of ET-dependent plant processes, including defense-related gene expression (Wang et al. 2002). Therefore, down regulation of the corresponding genes, as observed in WCS417r-treated roots, suggests that the onset of ISR is associated with a reduction in ET signaling. Among all of the WCS417r-responsive genes, the transcript for a flavonol reductase was observed as the most strongly induced. Flavonol reductases have been implicated in lignin and anthocyanin biosynthesis, for example (Ostergaard et al. 2001).

Although the roots clearly responded to colonization by WCS417r, we were unable to identify genes that showed a greater than twofold change in expression in the leaves of ISR-

Table 5. Fold-change ratios of induced systemic resistance (ISR)-specific *Pseudomonas syringae* pv. *tomato* DC3000-responsive genes in leaves of *P. fluorescens* WCS417r-treated plants

	Challenged leaves, fold-change ratio[a]			
Annotation[b]	**6 h**	**24 h**	**Probe set no.**	**AGI no.**
Transcription				
Hypothetical protein	3.4	2.5	13517_g_at	AT4G18690
WRKY family transcription factor	2.4	2.4	20382_s_at	AT2G30250
Similar to ethylene response factor 1	2.0	2.0	19755_at	AT2G31230
Squamosa promoter binding protein-related 2	–2.0	–2.8	18029_g_at	AT5G43270
Cell rescue and defense				
Putative thaumatin	2.3	2.0	19839_at	AT2G28790
Expressed protein	–2.0	–2.6	11995_at	AT2G29970
Regulation of and interaction with cellular environment				
TAT-binding protein, putative	–2.2	–2.1	14052_at	AT1G10070
Gluthatione reductase	–2.5	–2.0	13262_s_at	AT3G54660
Cellular communication or signal transduction				
Serine/threonine protein phosphatase type one (PP1)	6.1	5.5	20333_at	AT5G27840
Similar to receptor kinase 1	2.6	3.5	16348_at	AT1G65790
Calcium-dependent protein kinase (CDPK) (AK1)	–2.3	–2.4	16088_f_at	AT5G04870
Protein kinase 6-like	–2.5	–2.5	19917_at	AT5G58950
Protein serine/threonine kinase, putative	–2.6	–2.2	18316_at	AT1G01540
Metabolism				
NADC homolog	5.5	6.6	18657_at	AT2G01350
UDP-glycosyltransferase family	3.0	2.0	18512_at	AT1G24100
Cytochrome P450-like protein	2.4	2.6	12526_at	AT4G27710
Aspartate kinase-homoserine dehydrogenase	2.3	2.3	19749_at	AT1G31230
Pectinesterase-related	–2.3	–2.2	13635_at	AT4G12390
Energy				
Glyceraldehyde-3-phosphate dehydrogenase	–2.2	–2.5	20640_s_at	AT1G42970
Amine oxidase family	–2.6	–2.7	12241_at	AT4G29720
Protein with binding function or cofactor requirement				
Flowering time control protein (FCA)	2.7	2.1	13250_s_at	AT4G16280
26S protease regulatory subunit 6A	2.3	2.5	14026_at	AT1G09100
Transport facilitation				
CLC-c chloride channel protein	2.0	2.1	12493_g_at	AT5G49890
Protein fate (folding, modification, destination)				
Putative leucine aminopeptidase	2.1	2.1	17956_i_at	AT2G24200
Unclassified proteins				
Expressed protein	10.2	8.6	20165_at	AT1G23150
Expressed protein	2.9	2.4	19218_at	AT1G54520
Expressed protein	–2.0	–2.1	16499_at	AT4G32020
Expressed protein	–2.2	–2.1	19984_at	AT1G61900
Arabinogalactan-protein (AGP3)	–2.5	–2.9	15208_s_at	AT4G40090
Expressed protein	–2.8	–2.2	14450_at	AT1G79160

[a] Fold-change ratios (time = 6/0 or 24/0 h) are based on transcript profiles of leaves of ISR-expressing Col-0 plants at 0, 6, and 24 h after challenge inoculation with *P. syringae* pv. *tomato* DC3000. None of the selected genes showed a substantial change in expression in *P. syringae* pv. *tomato* DC3000-inoculated control plants.

[b] Annotations are as predicted by the MIPS *Arabidopsis thaliana* Genome Database.

expressing plants. None of the approximately 8,000 genes tested showed a consistent change in the level of expression in the aboveground plant parts, indicating that, in contrast to SAR, the onset of WCS417r-mediated ISR in the leaves is not associated with detectable changes in gene expression. Nevertheless, leaves from induced plants displayed a clearly enhanced resistance against a broad range of pathogens. In our experiments, the effectiveness of ISR was checked for *P. syringae* pv. *tomato* DC3000; however, under similar conditions, ISR also was demonstrated to be effective against *X. campestris* pv. *armoraciae*, *A. brassicicola*, *F. oxysporum* f. sp. *raphani*, and *Peronospora parasitica* (Pieterse et al. 1996; Ton et al. 2002b). To investigate the possibility that ISR is associated with transcriptional changes that are apparent only after pathogen attack, we analyzed the expression profile of the approximately 8,000 *Arabidopsis* genes in control and ISR-expressing plants upon challenge inoculation with *P. syringae* pv. *tomato* DC3000. Of the 425 *P. syringae* pv. *tomato* DC3000-responsive genes, 81 (19%) showed an augmented change in ISR-expressing leaves. Of this set of ISR-primed *P. syringae* pv. *tomato* DC3000-responsive genes, 63% showed an enhanced expression, whereas 37% were expressed exclusively in WCS417r-treated plants upon pathogen challenge. These results indicate that ISR-expressing plants are primed for augmented expression of a specific set of pathogen-responsive genes.

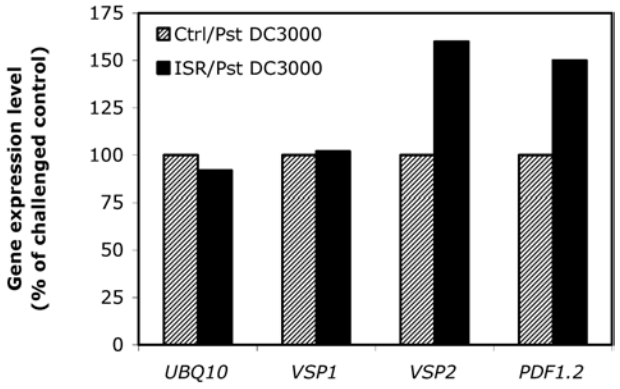

Fig. 3. Verification of leaf GeneChip data. Transcript levels of the *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000-responsive gene *VSP1*, the induced systemic resistance (ISR)-primed, *P. syringae* pv. *tomato* DC3000-responsive genes *VSP2* and *PDF1.2*, and the constitutively expressed gene *UBQ10* were analyzed in control and ISR-expressing plants at 24 h after inoculation with *P. syringae* pv. *tomato* DC3000. Hybridization signals obtained with the Northern blot analysis were quantified using a Phosphor Imager. Shown are relative transcript levels at 24 h after inoculation (mRNA levels in *P. syringae* pv. *tomato* DC3000-inoculated control plants were set at 100%).

Among the ISR-primed, pathogen-responsive genes, the majority of the genes were predicted to be influenced by JA or ET signaling, suggesting that both signals play an important role. These observations can explain our previous findings that, on the one hand, responsiveness to both JA and ET is required for full expression of ISR; whereas, on the other hand, ISR is not associated with enhanced production of these hormones prior to pathogen attack (Pieterse et al. 1998, 2000). Moreover, the expression profile data are in line with previous findings that WCS417r-treated plants are primed for enhanced expression of the JA-responsive *VSP2* gene and for augmented production of ET after challenge with *P. syringae* pv. *tomato* DC3000 (Hase et al. 2003; Van Wees et al. 1999). Hence, we postulate that colonization of the roots by ISR-inducing rhizobacteria triggers a primed state in systemic tissues, resulting in the augmented expression of specific JA- and ET-dependent defense responses upon pathogen challenge.

If this hypothesis is correct, one would expect ISR-expressing plants to exhibit an enhanced defensive capacity against pathogens that trigger, and are sensitive to, JA- or ET-dependent defenses. In a study in which the spectrum of effectiveness of rhizobacteria-mediated ISR was investigated, Ton and associates (2002b) recently demonstrated that, indeed, ISR is effective only against pathogens that are sensitive to JA- or ET-dependent basal defenses. It was postulated that ISR constitutes a reinforcement of extant JA- or ET-dependent basal defense responses, but the nature of this sensitizing effect remained unclear. Priming of JA- and ET-responsive genes allows ISR-expressing plants to respond faster or more strongly to pathogens that trigger JA- and ET-dependent defense responses; therefore, it is likely that this process plays an important role in the enhancement of JA- and ET-dependent basal defense responses during ISR.

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

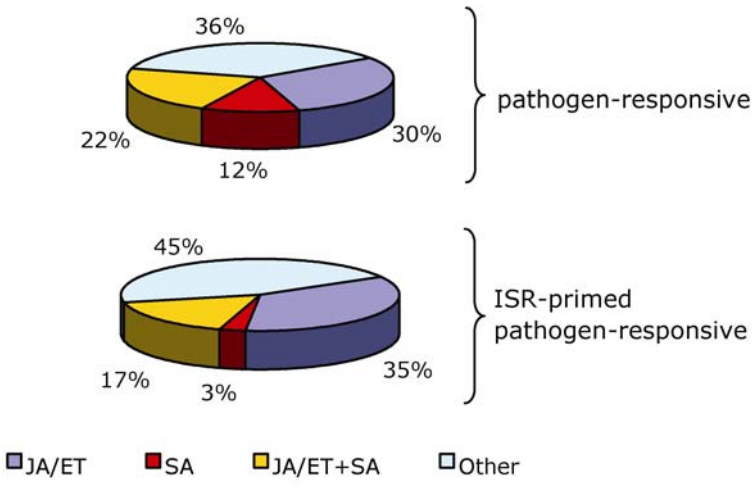

Fig. 4. The group of induced systemic resistance (ISR)-primed, pathogen-responsive genes is enriched for genes that are affected by jasmonic acid (JA) and ethylene (ET) signaling. The effect of JA, ET, and salicylic acid (SA) on pathogen-responsive gene expression was assessed for 254 *Arabidopsis* genes that showed a similar expression pattern in Col-0 plants in response to infection by *Pseudomonas syringae* pv. *tomato* DC3000 and *P. syringae* pv. *maculicola* ES4326. Shown are the frequency distributions of the pathogen-responsive genes over the classes JA- or ET-responsive (JA/ET), SA-responsive (SA), SA- and JA/ET-responsive (JA/ET+SA), and JA-, ET-, and SA-independent (other) for all the *P. syringae* pv. *tomato*- and *P. syringae* pv. *maculicola*-responsive genes and the group of pathogen-responsive genes that shows an augmented expression pattern in challenged ISR-expressing plants.

pathogen. In the past 10 years, priming has been associated with several types of induced resistance (Conrath et al. 2002). For instance, pretreatment of parsley cell cultures with low doses of the SAR activator SA, or its functional analogues 2,6-dichloroisonicotinic acid (INA) or benzothiodiazole (BTH), was demonstrated to prime the cells for potentiated activation of various cellular defense responses, in response to otherwise noninducing doses of a cell-wall elicitor from *Phytophthora sojae*. These potentiated responses included the early oxidative burst (Kauss and Jeblick 1995), the incorporation of various phenolics into the cell wall (Kauss et al. 1992), the secretion of antimicrobial phytoalexins (Katz et al. 1998; Kauss et al. 1992), and the potentiated expression of several defense-related genes (Thulke and Conrath 1998). In *Arabidopsis* and tobacco, SAR also has been shown to be associated with priming for potentiated expression of SA-responsive *PR* genes (Cameron et al. 1999; Kohler et al. 2002; Mur et al. 1996; Van Wees et al. 1999). Other types of induced resistance in which priming plays an important role are those triggered by the non-protein amino acid β-aminobutyric acid (BABA) (Ton and Mauch-Mani 2004; Zimmerli et al. 2000) and bacterial lipopolysaccharide (LPS) (Newman et al. 2002). In *Arabidopsis*, BABA pretreatment was shown to result in a more rapid and stronger deposition of callose-containing papillae at the site of infection by the oomycetous pathogen *Peronospora parasitica*, or to a strong potentiation of *PR-1* gene expression in response to infection by *Pseudomonas syringae* pv. *tomato* DC3000 (Zimmerli et al. 2000). In pepper plants, pretreatment with LPS resulted in accelerated synthesis of two antimicrobial hydroxycinnamoyl-tyramine conjugates and the potentiated expression of an acidic *PR-2* gene in response to infection by the bacterial pathogen *X. campestris* pv. *campestris* (Newman et al. 2002). A common feature of the different types of induced disease resistance is that they display effectiveness against different plant pathogens. It is tempting to speculate that the broad-spectrum characteristic of induced resistance is based on the conditioning of the tissue to react more effectively to an invading pathogen.

Recently, Cartieaux and associates (2003) performed a transcriptome analysis of *Arabidopsis* upon colonization of the roots by the rhizobacterium *P. thivervalensis* (strain MLG45). Like WCS417r, MLG45 induced a systemic resistance that was effective against P. syringae pv. tomato DC3000. However, in contrast to WCS417r, MLG45 induced very few changes in the transcriptome of roots; whereas, systemically in the leaves, an increase in defense-related transcripts was detected prior to challenge inoculation. These results clearly contrast with ours but can be explained readily. First of all, Cartieaux and associates (2003) performed their study in *Arabidopsis* accession Ws-0, which is blocked in its ability to express JA- and ET-dependent ISR, such as that triggered by WCS417r, due to a defect at the *ISR1* locus (Ton et al. 1999, 2001). By inference, WCS417r and MLG45 must induce different defense signaling pathways in *Arabidopsis*. Second, colonization of the roots by WCS417r stimulates plant growth in the absence of pathogen infection (Pieterse and Van Loon 1999). In contrast, MLG45 induced a clear growth reduction under such conditions (Cartieaux et al. 2003; Persello-Cartieaux et al. 2001), suggesting that the systemic changes in gene expression observed in MLG45-induced plants are likely to be caused by a more general stress response. One must conclude, therefore, that WCS417r and MLG45 induce different defense responses in *Arabidopsis.*

In conclusion, our study showed that the onset of WCS417r-mediated ISR is not associated with detectable changes in gene expression in leaves, but rather results from the induction of a primed state, allowing augmented expression of pathogen-responsive genes. The molecular mechanism of priming is still unclear. It is hypothesized that induction of the primed state results in an increase in the amount or activity of cellular components with important roles in defense signaling (Conrath et al. 2002). By itself, the increased presence or activity of these cellular signaling components have no major effect, but provide the plant with an enhanced capacity to respond to an invading pathogen. In terms of energy costs for the plant, priming might prove to be highly beneficial. On the one hand, the defense responses are expressed only when they are really needed (i.e., upon attack by a pathogen). On the other hand, only those defense responses are recruited that are triggered by the pathogen encountered. So what is the nature of the primed state of ISR? The lack of systemic changes in the expression of the approximately 8,000 *Arabidopsis* genes tested suggests that priming is not regulated at the transcriptional level, although crucial changes in gene expression might occur either below the level of detection or within the group of genes that was not present on the GeneChip. Alternatively, priming may be regulated post translationally. Either way, future research on the mechanism of priming will provide novel insights into how plants are able to defend themselves against harmful organisms.

MATERIALS AND METHODS

Growth conditions of rhizobacteria and plants.

Nonpathogenic *P. fluorescens* WCS417r bacteria were used for the induction of ISR. WCS417r was grown for 24 h at 28°C on King's medium B agar plates (King et al. 1954) containing the appropriate antibiotics as described previously (Pieterse et al. 1996). Subsequently, bacteria were collected and resuspended in 10 mM $MgSO_4$ to a density of 10^9 CFU/ml (optical density at 600 nm = 1.0) before being mixed through soil.

Seed of *Arabidopsis thaliana* accession Col-0 were sown in quartz sand. For isolation of RNA from roots, 2-week-old seedlings were transferred to a system of rock-wool cubes (Rockwool/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 (wt/vol) mixture of talcum powder and either a suspension of ISR-inducing WCS417r bacteria in 10 mM $MgSO_4$ (final density 5×10^8 CFU/g), or a solution of 10 mM $MgSO_4$ as a control. For isolation of RNA from leaves, 2-week-old seedlings were transferred to 60-ml pots containing a sand and potting soil mixture that had been autoclaved twice for 20 min with a 24-h interval. Before transfer of the seedlings, a suspension of ISR-inducing WCS417r bacteria (10^9 CFU/ml) was mixed through the soil to a final density of 5×10^7 CFU/g as described previously (Pieterse et al. 1996). Control soil was supplemented with an equal volume of 10 mM $MgSO_4$. Plants were cultivated in a growth chamber with a 9-h day (200 μE m^{-2} sec^{-1} at 24°C) and 15-h night (20°C) cycle at 70% relative humidity. Plants were watered once a week with water and once a week with modified half-strength Hoagland nutrient solution (Hoagland and Arnon 1938).

Pathogen inoculation and ISR bioassay.

The virulent bacterial pathogen *P. syringae* pv. *tomato* DC3000 (Whalen et al. 1991) was used for challenge inoculation. *P. syringae* pv. *tomato* DC3000 was grown overnight in liquid King's medium B at 28°C. Subsequently, bacterial cells were collected by centrifugation and resuspended to a final concentration of 2.5×10^7 CFU/ml in 10 mM $MgSO_4$ containing 0.015% (vol/vol) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands). To confirm expression of ISR in WCS417r-treated plants, ISR bioassays were performed as de-

scribed previously (Pieterse et al. 1996; Van Wees et al. 1997), using a subset of plants that were grown in parallel with the plants that were used for extraction of RNA. Briefly, 5-week-old plants were placed at 100% relative humidity 1 day prior to challenge inoculation. Subsequently, plants were inoculated by dipping the leaves for 2 s in a suspension of *P. syringae* pv. *tomato* DC3000 at 2.5×10^7 CFU/ml in 10 mM $MgSO_4$ and 0.015% (vol/vol) Silwet L-77. Four days later, disease severity was assessed by determining the percentage of diseased leaves per plant. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. The disease index was calculated for each plant ($n = 20$) based on the percentage of diseased leaves.

Sample preparation and microarray data collection.

For isolation of RNA from roots and noninoculated leaf tissues, whole root systems or entire rosettes were harvested 3 and 7 days after the start of the induction treatment (see above) and immediately frozen in liquid nitrogen. For extraction of RNA from *P. syringae* pv. *tomato* DC3000-inoculated leaves, whole rosettes from 5-week-old, soil-grown plants were harvested at 0, 6, and 24 h after challenge inoculation. RNA was prepared from frozen tissue using RNAwiz (Ambion, Huntington, U.K.). Subsequently, cRNA probe synthesis, hybridization to a GeneChip, and collection of data from the hybridized GeneChip was performed as described previously (Zhu et al. 2001). Hybridizations with labeled cRNAs were conducted with *Arabidopsis* GeneChip Microarrays (Affymetrix, Santa Clara, CA, U.S.A.) containing probe sets for approximately 8,000 *Arabidopsis* genes (Zhu and Wang 2000).

Expression profiling.

Expression data were normalized globally to the average value of 100 before data analysis. Genes with accurately detectable transcript levels were defined by probe sets showing averaged expression levels greater than 40, as described previously (Zhu and Wang 2000). For probe sets showing an expression value of <5, it was adjusted to 5. The fold changes between induction treatments and the respective controls were calculated by dividing the induction-treated expression values by the control expression values. Using these selection criteria for the identification of genes displaying greater than twofold change, we expected <0.25% false changes resulting from inaccuracies of hybridization and detection (Zhu and Wang 2000). Taking into account that, out of approximately 8,000 probe sets, only approximately 4,200 showed an expression level over 40, the number of probe sets showing false changes should be less than 11. To avoid false positives due to noise arising from variation in experimental conditions, we required the changes to be consistent in time, between experiments, or both.

RT-PCR and Northern blot analysis.

Total RNA was extracted by homogenizing frozen leaf tissue in extraction buffer (0.35 M glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% [wt/vol] sodium dodecyl sulfate, and plant tissue at 1 ml/g). The homogenates were extracted with phenol/chloroform/isoamylalcohol (25:24:1) and the RNA was precipitated using LiCl, as described by Sambrook and associates (1989). Analysis of gene expression in the roots was performed by RT-PCR, as described previously (Pieterse et al. 1998). Gene-specific primer pairs for the detection of *MYB72*, *MLO8*, and *VSP2* transcripts in the roots were designed based on the annotated sequences corresponding to AGI numbers AT1G56160 (probe set 12725_r_at), AT2G17480 (probe set 13687_s_at), and AT5G24770 (probe set 14675_s_at). Detection of the glucosyltransferase gene (probe set 17362_at, AGI number AT4G15260) and the "expressed protein" gene (probe set 18721_at, AGI number AT3G02040) was performed in a similar manner. 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 dimethyl sulfoxide, as described previously (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 primers based on the annotated sequences corresponding to AGI numbers AT4G05320 (*UBQ10*), AT5G24780 (*VSP1*), AT5G24770 (*VSP2*), and AT5G44420 (*PDF1.2*). After hybridization with α-^{32}P-dCTP-labeled probes, blots were exposed for autoradiography and signals quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, The Netherlands) with Quantity One software (BioRad).

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