Simultaneous Interaction of *Arabidopsis thaliana* with *Bradyrhizobium* Sp. Strain ORS278 and *Pseudomonas syringae* pv. *tomato* DC3000 Leads to Complex Transcriptome Changes

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Induced systemic resistance (ISR) is a process elicited by telluric microbes, referred to as plant growth-promoting rhizobacteria (PGPR), that protect the host plant against pathogen attacks. ISR has been defined from studies using *Pseudomonas* strains as the biocontrol agent. Here, we show for the first time that a photosynthetic *Bradyrhizobium* sp. strain, ORS278, also exhibits the ability to promote ISR in *Arabidopsis thaliana*, indicating that the ISR effect may be a widespread ability. To investigate the molecular bases of this response, we performed a transcriptome analysis designed to reveal the changes in gene expression induced by the PGPR, the pathogen alone, or by both. The results confirm the priming pattern of ISR described previously, meaning that a set of genes, of which the majority was predicted to be influenced by jasmonic acid or ethylene, was induced upon pathogen attack when plants were previously colonized by PGPR. The analysis and interpretation of transcriptome data revealed that 12-oxo-phytodienoic acid, an intermediate of the jasmonic acid biosynthesis pathway, is likely to be an actor in the signaling cascade involved in ISR. In addition, we show that the PGPR counterbalanced the pathogen-induced changes in expression of a series of genes.

Plants of which the roots are colonized by plant growth-promoting rhizobacteria (PGPR) can develop a form of protection of both under- and above-ground parts. This type of resistance is referred to as induced systemic resistance (ISR), in order to distinguish it from the systemic acquired resistance (SAR) developed after primary infection by a pathogen (Persello-Cartieaux et al. 2003). The existence of ISR was highlighted for the first time in the pathosystem *Dianthus caryophyllus*–*Fusarium oxysporum* sp. *dianthi*, whose roots were colonized by the nonpathogenic rhizobacteria *Pseudomonas fluorescens* WCS417r (Van Peer et al. 1991). A model based on *Arabidopsis*

was developed to study molecular bases of the induction of ISR by rhizobacteria (Van Loon et al. 1998). In this system, the strain *Pseudomonas fluorescens* WCS417r, known to induce ISR in carnation (Van Peer et al. 1991), radish (Leeman et al. 1995), tomato (Duijff et al. 1998), and *Arabidopsis* (Pieterse et al. 1996), was used as the protective agent. This protection is effective against various classes of pathogens, including bacterial leaf pathogens such as *Pseudomonas syringae* pv. *tomato* DC3000 and *Xanthomonas campestris* pv. *amoracia*, the fungal root pathogen *Fusarium oxysporum* sp. *raphani*, and the oomycete leaf pathogen *Peronospora parasitica* (Pieterse et al. 1996; Ton et al. 2002; Van Wees et al. 1997). Resistance to these pathogens appears by a delay in symptom development, a reduction of disease severity, and an inhibition of the pathogen growth. The spectrum of effectiveness of WCS417r-mediated ISR and pathogen-induced SAR overlaps but is also partly divergent (Ton et al. 2002). Moreover, the level of induced protection can be enhanced further when both ISR and SAR are activated simultaneously, indicating an additive effect of both type of resistances (Van Wees et al. 2000).

If SAR and ISR are phenotypically similar (a broad-spectrum systemic resistance), they differ by their molecular signature. The onset of SAR is unavoidably accompanied by a local and systemic increase in the endogenous level of salicylic acid (SA) (Ward et al. 1991). In contrast, ISR is independent of SA (Pieterse et al. 1996). Two types of molecular tools made it possible to precisely define the role of SA during the SAR: i) transgenic plants degrading SA (Gaffney et al. 1993) and ii) mutants unable to induce the biosynthesis of SA after an attack by a pathogen (*sid1-1* and *sid2-1* mutants; Nawrath and Métraux 1999). Using these mutant plants, it was also possible to show that the WCS417r-mediated ISR in *Arabidopsis* is independent of the accumulation of SA (Van Loon et al. 1998). On the other hand, the use of *jar1-1* and *etr1-1*, two mutants altered in the response to jasmonic acid (JA) and ethylene (ET) respectively, indicated that WCS417r-mediated ISR requires an intact signaling pathway to these two phytohormones (Pieterse et al. 1998).

SAR is characterized by the induction of a diverse array of plant proteins, including pathogenesis-related proteins (PR), while no ISR molecular markers have been identified so far. In order to identify genes potentially involved in ISR, three large-

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*The e-Xtra logo stands for “electronic extra” and indicates that Figure 1 and Table 1 appear in color online. Two supplemental tables are also published online.

scale analyses of the transcriptome have been carried out to date (Cartieaux et al. 2003; Verhagen et al. 2004; Wang et al. 2005). Two of these studies were performed without a challenging pathogen, which allowed identification of only a few defense-related genes, such as glutathione-S-transferase, endochitinase, or β -1,3-glucanase, to be differentially expressed in rhizobacteria-treated versus axenic plants (Cartieaux et al. 2003; Wang et al. 2005). The third analysis was designed to identify ISR-primed genes, i.e., genes of which expression was specifically modified in ISR-expressing plants that were further challenged by *Pseudomonas syringae* pv. *tomato* (Van Wees et al. 1999). The authors found 81 such ISR-primed genes among the 8,000 tested genes (one third of the *Arabidopsis* genome), including 19 that are responsive to JA or ET genes, or both, which confirmed the role of these two hormones throughout ISR onset.

A common point of almost all models designed so far to scrutinize plant-PGPR interactions was the use of pseudomonads or free-living nitrogen-fixing bacteria as bacterial partner. It appeared to us particularly relevant to extend the study of plant-PGPR interactions to another bacterial genus, since the question of the specificity of plant responses to different PGPR classes remains a matter of debate. The ORS278 strain is a unique photosynthetic *Bradyrhizobium* strain that is well-studied in our laboratory (Giraud et al. 2007). Inoculated to rice, it promotes root architecture changes together with a 10 to 20% yield increase (Chaintreuil et al. 2000). As this strain is distantly related to pseudomonads, it is an appealing candidate to check if the ability to promote ISR is universally distributed among rhizobacteria. Moreover, we wanted to test if an ORS278-mediated ISR would be comparable to the one elicited by WCS417r. Therefore, we decided to conduct our work using *Arabidopsis* as a model system, together with *Pseudomonas syringae* pv. *tomato* as the challenge pathogen. The experimental plan designed to investigate the genome-wide expression profiling of ISR-expressing plants using CATMA microarrays (Hilson et al. 2004) included four types of samples: i) control plants in which leaves were infiltrated with sterile MgSO_4 , ii) plants in which roots were colonized with ORS278 and leaves infiltrated with sterile MgSO_4 , iii) plants in which leaves were infiltrated with pathogens, and iv) plants in which roots were colonized with ORS278 and leaves infiltrated with pathogens. Five cross-hybridizations were realized on CATMA slides: plants in group i crossed with those in ii, iii, or iv, and those in group iv crossed with those in groups ii or iii. The results indicate that a component of the oxylipin biosynthesis pathway was involved in ISR onset. Furthermore, this study revealed that rhizospheric *Bradyrhizobium* sp. strain ORS278 alleviated transcriptional changes induced by leaf-inoculated *P. syringae* pv. *tomato* DC3000, in particular the induction of defense-related genes and the repression of photosynthesis genes.

RESULTS

Bradyrhizobium-mediated ISR against *P. syringae*.

The dry weight of shoots of *in vitro* two-week-old *Arabidopsis* plants measured one week after *Bradyrhizobium* sp. strain ORS278 inoculation (an average of 0.329 mg per shoot, $n = 18$) was significantly greater than in control plants (0.259 mg per shoot, $n = 18$). Therefore, *Bradyrhizobium* sp. strain ORS278 appears to promote *Arabidopsis* growth similarly to what has been shown in rice (Chaintreuil et al. 2000).

Since the protection against pathogens has been reported to be a core component of plant growth-promoting ability of PGPR (Persello-Cartieaux et al. 2003), we tested the protective property of *Bradyrhizobium* sp. strain ORS278 by challenging the bacterized *Arabidopsis* with *P. syringae* pv. *tomato* DC3000,

a bacterial pathogen causing leaf speck disease on *Arabidopsis* (Whalen et al. 1991). Seven days after infection with *P. syringae*, control plants displayed severe disease symptoms (Fig. 1A). In contrast, plants in which roots had previously been colonized by *Bradyrhizobium* sp. strain ORS278 showed necrotic lesions that resemble those induced during incompatible plant-pathogen interactions. Consistent with this observation, the treatment with *Bradyrhizobium* sp. strain ORS278 resulted in inhibition of growth of *P. syringae* by up to sevenfold on day 4 after inoculation as compared with control plants (Fig. 1B). In the culture conditions used in this experiment, there was no significant difference between the shoot fresh weights of ORS278-protected and control plants (85 ± 31 and 78 ± 29 mg of FW, respectively) and the leaf numbers of either type of plant (11.0 ± 0.9 and 10.7 ± 1.1 , respectively). Therefore, the ORS278-induced resistance cannot be attributed to a developmentally regulated process, such as the age-related resistance described elsewhere (Murphy et al. 2003). Overall, our results thus indicate that *Bradyrhizobium* sp. strain ORS278 has the ability to induce effective systemic resistance against infection with *P. syringae* pv. *tomato* DC 3000.

Metabolite fingerprinting experiments demonstrate minor within-replicate sample variability compared with predominant between-treatment variation.

Metabolomics can be used to profile and identify marker metabolites, which change in pool size upon treatments (metabolite profiling) (Broeckling et al. 2005; Colebatch et al. 2004; Desbrosses et al. 2005). In addition, this approach is highly useful for assessing within-replicate variability compared

Fig. 1. *Bradyrhizobium* sp. strain ORS278 colonization triggered effective systemic resistance against challenge infection of *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis* plants. **A**, Disease symptoms on plants 7 days after inoculation with *P. syringae* pv. *tomato* DC3000. **B**, In planta growth of *P. syringae* pv. *tomato* DC3000 in control and ORS278-inoculated plants. Concentrations of bacteria in leaves of control (open bars) and ISR-expressing plants (gray bars) were assayed 0 and 4 days after manual infiltration with a 2×10^5 CFU/ml suspension. Data points are means (CFU/g) with standard errors from 30 infected leaves. The asterisk indicates statistically significant differences between treatments (Student's *t* test, $\alpha \leq 0.05$). Similar results (data not shown) were obtained in a second and third independent experiments.

Since this experiment had been designed to identify genes involved in the onset of ORS278-mediated ISR, our samples consisted of ISR-expressing plants challenged by *P. syringae* pv. *tomato* DC3000. Infected leaves were collected 24 h after infection by the pathogen. Gas chromatographic-electrospray ionization-time of flight-mass spectrometry (GC-EI-TOF-MS)-based metabolite fingerprints were performed on each of the samples issued from three leaves harvested on three different plants obtained through three independent cultures corresponding to three independent biotic treatments. The GC-MS-based metabolite fingerprinting and data normalization was essentially performed as described by Desbrosses and associates (2005) and was applied to all available nonpooled independent replications comprising nine replications per biological treatment (Supplementary Table 1). We performed two independent statistical approaches for sample classification. Hierarchical clustering analysis (HCA)-based Euclidian distance clearly demonstrated unambiguous classification of replicate samples into separate branches of the clustering tree (Fig. 2). No missorting was observed. However, because missing value substitution and manual elimination of mass fragments representing laboratory contaminations or internal standards may bias clustering analyses, we performed an alternative investigation. Independent component analysis (ICA) in combination with applying analysis of variance (ANOVA) for filtering nonrelevant mass fragments confirmed our initial finding. In addition, it demon-

In planta pathogen growth was not significantly different in ORS278-protected and control plants at this timepoint (7.93 ± 0.53 and 7.49 ± 0.32 log CFU g⁻¹ FW respectively). Therefore, the metabolite profiling modifications observed are most likely related to PGPR response rather than to a modulation of the pathogen response. From the statistical analyses performed, we concluded that our experiment consisted of three validated biological repeats, and we decided to pool the samples within each biological treatment for our transcriptome analysis.

Since the biological replications were highly homogeneous, we pooled the nine biological samples corresponding to each treatment. Total RNA prepared from these pooled leaf samples collected from control and treated plants were then analyzed using the CATMA chips. The CATMA project (complete *Arabidopsis* transcriptome microarray) provides the *Arabidopsis* scientific community with a generic array to address all biological questions concerning this species. The current version of CATMA contains 24,576 probes, representing about 85% of the genes predicted by Eugene (Foissac et al. 2003). The probes are 'gene sequence tags' (GST) designed with specific primers and amplicons design software (Thareau et al. 2003).

○ MgSO4
● ORS278 + MgSO4
▨ Pst
● ORS278+Pst

IC2

IC1

Fig. 4. Impact of *Bradyrhizobium* sp. strain ORS278 on its host metabolism. **A**, The MapMan Metabolism overview display created using the 203 *Bradyrhizobium* sp. strain ORS278-regulated genes identified from the plant growth-promoting rhizobacteria versus control comparison. **B**, The MapMan cellular response overview display created using the same pool of genes. The fold change is displayed as illustrated in the fold change color bar in the upper left of each panel (red is repressed, blue is induced).

are predicted to be involved in metabolism, 12% in cellular transport, 10% in signal transduction, 5% in protein metabolism, 3% in regulation of transcription, 3% in cell wall modification, and 3% in defense response. Among all the *Bradyrhizobium* sp. strain ORS278-responsive genes, a gene coding for an aconitase (enzyme involved in the Krebs' cycle) was observed as the most strongly repressed (i.e., sevenfold). To draw a comprehensive overview of the cellular and metabolism responses of *Arabidopsis*, we took advantage from the Mapman toolbox developed by Thimm and coworkers (2004). The MapMan overview metabolism map highlights the fact that genes involved in biotic and abiotic stress are down-regulated (Fig. 4).

An earlier transcriptome analysis showed no detectable increase in transcript level associated to ISR (Verhagen et al. 2004). However, the same study highlighted a set of 30 genes whose expression was modified after *P. syringae* pv. tomato DC3000 challenge in ISR-expressing plants but not when plants were challenged only by the pathogen. The authors referred to these genes as primed ISR-specific. We observed the same priming pattern in our set of experiments: 64 genes showed an increased level of transcription in ISR-expressing plants after pathogen attack as compared with that in control plants (Table

1, Figs. 5B and D). The majority of the 64 primed ISR-specific genes belonged to the following functional categories: metabolism (23%), defense response (11%), regulation of transcription (9%), signal transduction (9%), and cellular transport (6%). Among the seven genes annotated as 'defense response,' *PDF1.1*, a putative defensin, was overexpressed in response to *Bradyrhizobium* sp. strain ORS278 colonization independently of pathogen attack. This is the only gene that showed an upregulation of its transcription after *Bradyrhizobium* sp. strain ORS278 colonization among the approximately 22,000 genes represented on the CATMA array. In addition to the seven primed ISR-specific genes annotated as 'defense response,' three genes annotated as 'metabolism' (AT1G19670, AT4G16260, AT5G05730) have been reported to be induced by jasmonate, which tends to demonstrate that they could be involved in response to wounding or pathogen attack. Seven transcription factors annotated as 'regulation of transcription' are all involved in response to biotic or abiotic stresses, and five of them are actually related to response to wounding or pathogen attacks. Other genes related to elevated disease resistance could be genes coding for heat shock proteins (AT3G09440, AT5G02500) and the PEN3/PDR8 ATP-binding

Table 1. Fold-change ratios of induced systemic resistance (ISR)-primed *Pseudomonas syringae* pv. tomato DC3000-responsive genes in leaves of *Bradyrhizobium* ORS278-treated plants^a

		ORS278- <i>Pseudomonas syringae</i> pv. <i>tomato</i> /control					
		ORS278		<i>Pseudomonas syringae</i> pv. <i>tomato</i> /control		<i>Pseudomonas syringae</i> pv. <i>tomato</i> /control	
AGI name	Function TIGR	Fold-change	P-value	Fold-change	P-value	Fold-change	P-value
Defense response							
AT1G75830	Plant defensin-fusion protein, putative (PDF1.1)	1.50	0.00E + 0	1.63	0.0E + 00	-1.53	0.00E + 0
AT2G37040	Phenylalanine ammonia-lyase 1 (PAL1)	-0.54	1.00E + 0	1.36	0.0E + 00	-0.13	1.00E + 0
AT3G26200	Cytochrome P450 71B22, putative (CYP71B22)	0.50	1.00E + 0	1.44	0.0E + 00	0.50	1.00E + 0
AT4G19120	Early-responsive to dehydration stress protein (ERD3)	-0.96	9.98E - 5	1.02	3.5E - 06	-0.59	1.00E + 0
AT4G23100	Gamma-glutamylcysteine synthetase (GSH1)	-1.50	0.00E + 0	1.35	0.0E + 00	0.28	1.00E + 0
AT5G40170	Disease resistance family protein	-1.07	1.21E - 6	1.34	0.0E + 00	-0.29	1.00E + 0
AT5G45340	Cytochrome P450 family protein	0.15	1.00E + 0	1.08	1.9E - 07	0.69	5.59E - 1
Metabolism							
AT1G03310	Isoamylase, putative/starch debranching enzyme, putative	-0.66	1.00E + 0	1.01	4.7E - 06	-0.46	1.00E + 0
AT1G19670	Coronatine-responsive protein (COR1)	0.59	1.00E + 0	1.39	0.0E + 00	-0.26	1.00E + 0
AT1G50480	10-formyltetrahydrofolate synthetase (THFS)	-1.56	0.00E + 0	1.09	1.3E - 7	-0.82	1.21E - 2
AT2G16500	Arginine decarboxylase 1 (SPE1) (ARGDC)	-2.14	0.00E + 0	1.23	2.2E - 10	-0.28	1.00E + 0
AT2G29630	Thiamine biosynthesis family protein/thiC family protein	-3.91	0.00E + 0	1.57	0.0E + 00	-1.69	0.00E + 0
AT3G13750	Beta-galactosidase, putative/lactase, putative	-0.04	1.00E + 0	1.04	1.4E - 06	-0.04	1.00E + 0
AT3G46970	Starch phosphorylase, putative	-2.85	0.00E + 0	1.22	4.3E - 10	-1.19	1.00E - 8
AT3G48560	Acetolactate synthase, chloroplast (ALS)	-1.74	0.00E + 0	1.12	3.1E - 08	-0.43	1.00E + 0
AT3G55410	Oxoglutarate decarboxylase, putative	-0.04	1.00E + 0	1.05	9.2E - 07	-0.16	1.00E + 0
AT4G04610	PAPS reductase homolog (PRH19)	-2.46	0.00E + 0	1.04	1.4E - 06	-0.06	1.00E + 0
AT4G16260	Glycosyl hydrolase family 17 protein	-0.18	1.00E + 0	1.45	0.0E + 00	0.68	7.52E - 1
AT5G04590	Sulfite reductase/ferredoxin (SIR)	-1.59	0.00E + 0	1.08	2.2E - 07	-0.62	1.00E + 0
AT5G05730	Anthranilate synthase, alpha subunit (ASA1)	-1.32	1.12E - 11	1.99	0.0E + 00	0.49	1.00E + 0
AT5G42270	Ftsh protease, putative	-0.61	1.00E + 0	1.12	3.8E - 08	-0.37	1.00E + 0
AT5G49720	Endo-1,4-beta-glucanase KORRIGAN (KOR)	-2.63	0.00E + 0	1.19	1.6E - 09	-0.57	1.00E + 0
Protein folding							
AT3G09440	Heat shock cognate 70 kDa protein 3 (HSC70-3)	-3.27	0.00E + 0	1.9	0.0E + 00	-0.18	1.00E + 0
AT5G02500	Heat shock cognate 70 kDa protein 1 (HSC70-1)	-1.26	2.57E - 10	1.40	0.0E + 00	0.05	1.00E + 0
AT5G42020	Luminal binding protein 2 (BiP-2) (BP2)	-4.49	0.00E + 0	1.23	1.7E - 10	-0.58	1.00E + 0
DNA metabolism							
AT2G42520	DEAD box RNA helicase, putative	-0.63	1.00E + 0	1.06	5.9E - 07	-0.15	1.00E + 0
AT5G08610	DEAD box RNA helicase (RH26)	-1.79	0.00E + 0	1.73	0.0E + 00	-0.82	1.36E - 2
Signal transduction							
AT1G03740	Protein kinase family protein	-0.96	1.02E - 4	1.24	1.1E - 10	0.02	1.00E + 0
AT1G15690	Pyrophosphate-energized inorganic pyrophosphatase	-0.32	1.00E + 0	1.62	0.0E + 00	-0.70	4.63E - 1
AT1G73500	Mitogen-activated protein kinase kinase (MAPKK)	0.17	1.00E + 0	1.14	1.4E - 08	0.25	1.00E + 0

Continued on following page

^a Plants were four weeks old and all samples used for RNA extraction were collected 24 h after infection. Genes in which expression has been confirmed by real-time polymerase chain reaction are indicated in bold.

cassette transporter involved in nonhost resistance (AT1G59870; Stein et al. 2006). Finally, altogether, genes potentially involved in elevated disease resistance represent 24% of the primed ISR-specific genes found in our PGPR pathosystem.

The MapMan overview maps show that inoculation with ORS 278 resulted in decreased responsiveness of overall gene expression changes triggered by *P. syringae* pv. tomato DC3000 attack (Fig. 5A and C as compared with B and D).

Quantitative polymerase chain reaction (qPCR) analysis of representative primed ISR-specific genes.

To validate the results obtained with the CATMA microarray, the expression pattern of seven clones (AT1G75830, AT2G37040, AT1G19670, AT4G16260, AT5G05730, AT1G27730, and AT1G59870), selected on the basis of their ISR-specific upregulation, was further analyzed by qPCR (Fig. 6). For this purpose, RNA extracted from untreated and treated plants from three independent biological experiments were analyzed independently. For six genes tested (ABC transporter [AT1G59870], *AtCLH1*, *ZAT10*, *PAL1*, β 1.3 glucanase [AT4G16260], and *ASA1*), the analysis performed fully confirmed the results obtained in the DNA microarray hybridization experiments, i.e.,

an increased expression in plants expressing ISR after pathogen attack (primed ISR-specific genes). On the contrary, the upregulation of the *PDF1.1* gene following *Bradyrhizobium* sp. strain ORS278 colonization observed with the CATMA microarrays was not confirmed by qPCR analysis (Fig. 6B). As *PDF1.1* belongs to a multigene family, we suspected that this discrepancy could be due to a nonspecific hybridization of cDNAs of other members of the family on CATMA microarrays. This possibility has been highlighted by CATMA GST designers through a code used to classify GST according to their quality and available on FLAGdb database (Samson et al. 2004). In order to test this hypothesis of a cross-hybridization, we aligned the sequence of the *PDF1.1* gene-specific sequence tag (GSTpdf1.1) spotted on CATMA microarrays against the sequences of *PDF1.1*, *PDF1.2a*, *PDF1.2b*, *PDF1.2c*, and *PDF1.3* (Fig. 7). The six sequences shared a 40-bp consensus, which could lead to a nonspecific hybridization of *PDF1.2a*, *PDF1.2b*, *PDF1.2c*, and *PDF1.3* cDNAs on GSTpdf1.1. To clearly identify the genes whose expression was induced by *Bradyrhizobium* sp. strain ORS278, we designed specific pairs of primers for the seven members of the *PDF1* family (Table 2). The results of the qPCR analyses performed with those

Table 1. Continued from preceding page

		ORS278- <i>Pseudomonas syringae</i> pv. <i>tomato</i> /control					
		ORS278		<i>Pseudomonas syringae</i> pv. <i>tomato</i> /control		<i>Pseudomonas syringae</i> pv. <i>tomato</i> /control	
AGI name	Function TIGR	Fold-change	P-value	Fold-change	P-value	Fold-change	P-value
Signal transduction (<i>Continued</i>)							
AT1G79680	Wall-associated kinase, putative	-0.38	1.00E + 0	1.08	2.1E - 07	0.86	4.40E - 3
AT3G45860	Receptor-like protein kinase, putative	-1.31	1.68E - 11	1.26	5.6E - 11	-0.25	1.00E + 0
AT4G00720	Shaggy-related protein kinase theta/ASK-theta (ASK8)	-1.66	0.00E + 0	1.21	4.3E - 10	-0.03	1.00E + 0
Ubiquitin-dependent protein catabolism							
AT1G68050	F-box family protein (FKF1)/adagio 3 (ADO3)	-1.15	2.96E - 8	1.05	7.2E - 07	-0.31	1.00E + 0
AT2G25490	F-box family protein (FBL6)	-1.02	1.00E - 5	2.26	0.0E + 00	-0.09	1.00E + 0
AT5G15400	U-box domain-containing protein	-0.21	1.00E + 0	1.22	2.9E - 10	-0.06	1.00E + 0
Regulation of transcription							
AT1G27730	Zinc finger (C2H2 type) family protein (ZAT10)	0.04	1.00E + 0	1.21	4.6E - 10	0.44	1.00E + 0
AT1G68840	AP2 domain-containing protein RAP2.8	0.76	7.56E - 2	1.29	5.6E - 12	-0.04	1.00E + 0
AT4G23810	WRKY53 family transcription factor	0.47	1.00E + 0	1.04	1.2E - 06	0.23	1.00E + 0
AT4G36990	Heat shock transcription factor 4 (HSTF4)	-0.57	1.00E + 0	1.01	4.6E - 06	0.56	1.00E + 0
AT5G04340	Zinc finger (C2H2 type) family protein	0.24	1.00E + 0	1.00	6.0E - 06	-0.10	1.00E + 0
AT5G67450	Zinc finger (C2H2 type) protein 1 (AZF1)	0.54	1.00E + 0	1.07	3.8E - 07	0.41	1.00E + 0
Cellular transport							
AT1G59870	ABC transporter family protein	-2.67	0.00E + 0	1.82	0.0E + 00	-0.13	1.00E + 0
AT2G21390	Coatomer protein complex, subunit alpha, putative	-0.04	1.00E + 0	1.09	1.3E - 07	-0.09	1.00E + 0
AT2G22500	Mitochondrial substrate carrier family protein	0.29	1.00E + 0	1.36	0.0E + 00	-0.16	1.00E + 0
AT2G46430	Cyclic nucleotide-regulated ion channel (CNGC3)	-1.61	0.00E + 0	1.05	8.4E - 07	-0.07	1.00E + 0
Unclassified protein							
AT1G43690	Ubiquitin interaction motif-containing protein	-1.51	0.00E + 0	1.05	7.8E - 07	-0.25	1.00E + 0
AT1G51980	Mitochondrial processing peptidase alpha subunit, putative	-1.89	0.00E + 0	1.28	1.7E - 11	0.02	1.00E + 0
AT1G70700	Expressed protein	0.06	1.00E + 0	1.66	0.0E + 00	0.32	1.00E + 0
AT1G79600	ABC1 family protein	-0.30	1.00E + 0	1.03	2.3E - 06	-0.17	1.00E + 0
AT2G20960	Expressed protein	-1.00	1.73E - 5	1.56	0.0E + 00	0.23	1.00E + 0
AT2G31460	Hypothetical protein	-3.61	No	1.36	0.0E + 00	-0.52	1.00E + 0
AT2G40000	Expressed protein	-0.32	1.00E + 0	1.38	0.0E + 00	0.49	1.00E + 0
AT2G45220	Pectinesterase family protein	-1.89	0.00E + 0	1.27	3.4E - 11	0.07	1.00E + 0
AT3G07700	ABC1 family protein	-2.27	0.00E + 0	1.24	1.1E - 10	-0.14	1.00E + 0
AT3G09020	Alpha 1,4-glycosyltransferase family protein	-0.03	1.00E + 0	1.04	1.5E - 06	0.26	1.00E + 0
AT3G19030	Expressed protein	0.93	2.76E - 4	1.27	3.4E - 11	0.15	1.00E + 0
AT3G27260	DNA-binding bromodomain-containing protein	-0.55	1.00E + 0	1.12	4.5E - 08	-0.36	1.00E + 0
AT3G33004	Pseudogene, DNA-directed RNA polymerase beta-chain	-0.07	1.00E + 0	1.08	2.1E - 07	-0.41	1.00E + 0
AT3G48650	Pseudogene, At14a-related protein	-0.19	1.00E + 0	1.56	0.0E + 00	0.55	1.00E + 0
AT3G60750	Transketolase, putative	-3.15	0.00E + 0	2.06	0.0E + 00	-1.50	0.00E + 0
AT4G12290	Copper amine oxidase, putative	-1.97	0.00E + 0	1.41	0.0E + 00	-0.39	1.00E + 0
AT4G32020	Expressed protein	-0.30	1.00E + 0	1.13	2.3E - 08	-0.14	1.00E + 0
AT4G38770	Proline-rich family protein (PRP4)	-1.30	2.80E - 11	1.54	0.0E + 00	-0.92	4.05E - 4

Fig. 5. *Bradyrhizobium* sp. strain ORS278 counterbalances the impact of *Pseudomonas syringae* pv. tomato pathogenesis on host metabolism. **A**, The MapMan cellular response overview display, created using the 707 *Pseudomonas syringae* pv. tomato-regulated genes identified from the *P. syringae* pv. tomato DC3000-challenged plants versus control comparison. **B**, The MapMan cellular response overview display created using the 151 induced systemic resistance (ISR)-regulated genes identified from the ORS278-colonized and *P. syringae* pv. tomato DC3000-challenged plants versus control comparison. **C**, The MapMan metabolism overview display created using the 707 *Pseudomonas syringae* pv. tomato-regulated genes identified from the *P. syringae* pv. tomato DC3000-challenged plants versus control comparison. **D**, The MapMan metabolism overview display created using the 151 ISR-regulated genes identified from the ORS278-colonized and *P. syringae* pv. tomato DC3000-challenged plants versus control comparison. The fold change is displayed as illustrated in the fold change color bar in the upper left of each panel (red is repressed, blue is induced).

Fig. 5. Continued from preceding page.

primers are shown in Figure 6B. The expression of *PDF1.1*, *PDF1.4*, and *PDF1.5* genes was not changed in any condition tested. By contrast, *PDF1.2b*, *PDF1.2c*, and *PDF1.3* were up-regulated upon inoculation with *Bradyrhizobium* sp. strain ORS278 in the absence of pathogen challenge. *PDF1.2a* exhibited a typical primed ISR-specific gene expression pattern. According to these results, we assume that the pattern of expression revealed by CATMA experiments reflected the sum of *PDF1.2* and *PDF1.3* expressions.

DISCUSSION

In the present study, a global analysis of leaf transcript modifications associated with the *Arabidopsis*–*Bradyrhizobium* sp. strain ORS278 interaction in relation to responses to a leaf

pathogen was performed using CATMA DNA chips (Crowe et al. 2003; Hilson et al. 2004).

Since we were interested in identifying components of the signaling cascades leading to ISR onset and additional targets of these cascades, it appeared to us mostly appropriate to investigate in parallel changes in transcription induced by *Bradyrhizobium* sp. strain ORS278 in *Arabidopsis* and those induced by *Bradyrhizobium* sp. strain ORS278 in *P. syringae* pv. tomato DC3000–challenged *Arabidopsis*. Actually previous studies performed either with plant growth-promoting fungi or PGPR tend to demonstrate that priming is a common feature of the resistance responses induced by beneficial microorganisms (Conrath et al. 2006). For all these reasons, we chose to compare four classes of samples: control leaves, ORS278-protected plant leaves, *P. syringae* pv. tomato DC3000–challenged plant

Fig. 6. Expression pattern of the ABC transporter [AT1G59870], *AtCLH1*, *ZAT10*, *PAL1*, β-1,3-glucanase [AT4G16260], *ASA1*, and *PDF1s* genes in plants expressing induced systemic resistance (ISR) mediated by *Bradyrhizobium* sp. strain ORS278. The gene expression levels were quantified by real-time reverse transcriptase-polymerase chain reaction. Three treatments are compared: i) control plants in which leaves were infiltrated with sterile MgSO_4 solution, ii) plant growth-promoting rhizobacteria plants cocultivated with *Bradyrhizobium* sp. strain ORS278 and infiltrated with sterile MgSO_4 solution, and iii) ISR plants cocultivated with *Bradyrhizobium* sp. strain ORS278 and infiltrated with a 2×10^5 CFU/ml suspension of *Pseudomonas syringae* pv. tomato DC3000. Plants were 4 weeks old, and all samples were collected 24 h after infection. **A**, ABC transporter [AT1G59870], *AtCLH1*, *ZAT10*, *PAL1*, β-1,3-glucanase [AT4G16260], and *ASA1* expression level in shoots of control and treated plants is expressed relatively to the constitutive *AtACT2* gene expression level. **B**, *PDF1* subfamily gene expression level in shoots of control and treated plants. Average $\pm$ standard error from three independent experiments (biological variability) are given.

leaves, and protected-challenged plant leaves. Five cross-hybridizations were performed: ORS278-protected plants versus control leaves, *P. syringae* pv. tomato DC3000-challenged plants versus control leaves and protected-challenged plants versus, respectively, control leaves, ORS278-protected leaves, and *P. syringae* pv. tomato DC3000-challenged leaves.

Since metabolic changes are in part correlated to transcriptional changes, we extracted soluble metabolites and performed a metabolite fingerprinting analysis to determine if it was or was not convenient to pool our samples before CATMA hybridization. This metabolome fingerprinting showed that biological replications were highly homogeneous and allowed us to pool the nine biological samples corresponding to each treatment.

***Bradyrhizobium* sp. strain ORS278 down-regulates host gene expression.**

The CATMA data study (Fig. 4) showed that: i) 203 of the 22,089 genes tested displayed a downregulation of their expression in response to colonization by *Bradyrhizobium* sp.

strain ORS278, and ii) none of them showed an upregulation, except for the PDF1.2 genes subfamily. The shift in the expression of 203 genes detected in leaves indicates that the presence of *Bradyrhizobium* sp. strain ORS278 on *Arabidopsis* roots could be perceived systemically in leaves. It is noteworthy that the expression of almost all these genes was down-regulated. This trend is consistent with a previous large-scale transcriptome analysis of *Arabidopsis*-*P. fluorescens* WCS417r interaction, which showed that the majority of 97 genes responding to ISR-inducing rhizobacteria were actually down-regulated (Verhagen et al. 2004). Contrary to our study, in the case of *Pseudomonas fluorescens* WCS417r-mediated ISR, gene expression levels were recorded in the roots and not in the leaves, and the microarrays used represented only a third of the *Arabidopsis* genome. Transcriptome analyses of *Arabidopsis* colonized by *Pseudomonas thivervalensis* MLG45 or *Pseudomonas fluorescens* FPT9601-T5, respectively, showed equivalent number of genes up- and downregulated upon PGPR inoculation (Cartieaux et al. 2003; Wang et al. 2005). The apparent discrepancy between the present data and those previously obtained in

Fig. 7. GSTpdf1.1, the *PDF1.1* gene-specific sequence tag spotted on CATMA microarray. The GSTpdf1.1 sequence shared a 40-bp consensus with the sequences of four members of the PDF1 subfamily, *PDF1.2a*, *PDF1.2b*, *PDF1.2c*, and *PDF1.3*. Conserved amino acids are highlighted.

Table 2. Gene specific primer sets for real-time reverse transcriptase-polymerase chain reaction amplification

AGI Code	Name	Forward primer	Reverse primer	Product size (bp)
At2g37040	PAL1	ACCAAAAACGGTGTGCGACTT	GCGGAGTGTGGCAATGTGTG	101
At4g16260		AGTGGAAGTACAGTAGGTGTATG	GGCTTGGTTTGGATCGTAGAGT	126
At1g19670	AtCHL1	AAGTGGAAGTGGACGATGC	CCGAAGTTGGTAGGTGAGC	75
At1g59870	PEN3	TCGTGTACCAAGGACCGAGAGA	CTTTCTTGGAAAGTAACCTCTTGCAG	114
At1g27730	ZAT10	GTGTCCAACCTCCGAAGGTGC	TTGACCATCGAGAATTTCAGGG	101
At5g05730	ASA1	GCGTTGGTCTGTATAGCGTTG	TGGGATCTCCATTGGATCTTCCG	134
At1g75830	PDF1,1	GCTAAGTCTGCTACCATCTTAC	TGCTTCCACCAACCATCGG	84
AT5G44420	PDF1,2a	CTTGTTCTCTTTGCTGCTTTCGAC	ATGCATTACTGTTTCCGCAAACC	106
AT2G26020	PDF1,2b	TTCCATCATCACCTTTATCTACG	CCGCAAACTCCTGACCAAGTA	120
AT5G44430	PDF1,2c	CTGCTACCATCATCACCTTCC	CCGCAAACTCCTGACCAAGTA	126
AT2G26010	PDF1,3	GCCATCATCACTTTCTCTTTC	TTCTGTGCTTCCACCATATC	77
AT1G19610	PDF1,4	GATGATGGCGGTGGAAGGAAG	CGGGAAGTGAAGCGTGACAAG	139
AT1G55010	PDF1,5	TAACAATGATAGAGGAAATGATGG	TTAGCAAGAGAAGAAACAGAAAC	82
AT3G18780	ACT2	TCCCTCAGCACATTCCAGCAGAT	AACGATTCTGGACCTGCCTCATC	69

the MLG45-*Arabidopsis* interaction is most likely due to the fact that the previous study was performed in the *Arabidopsis* accession Ws-0, which is known to be blocked in its ability to express JA- and ET-dependent ISR, such as that triggered by *P. fluorescens* WCS417r (Ton et al. 1999, 2001). By inference, *P. thivervalensis* MLG45 is likely to induce in *Arabidopsis* a signaling pathway different from those induced by *P. fluorescens* WCS417r or *Bradyrhizobium* sp. strain ORS278. Likewise, *Pseudomonas fluorescens* FPT9601-T5-mediated protection might be achieved through alternative pathways, since this ability has been partly linked to production of antibiotics, siderophores, and proteases (Wang et al. 2005).

A MapMan overview metabolism map revealed that changes in expression induced by *Bradyrhizobium* sp. strain ORS278 colonization were completely different from the pathogen signature (Figs. 4 and 5A and C). Challenging *Arabidopsis* with *P. syringae* pv. tomato DC3000, as previously pointed out by Thilmony and coworkers (2006), leads to a significant repression of photosynthetic genes, coupled with an induction of defense-related genes (Fig. 5A). Conversely, colonization of *Arabidopsis* roots by *Bradyrhizobium* sp. strain ORS278 is illustrated by a weak impact on photosynthetic genes and by the repression of some genes involved in response to biotic and abiotic stresses, including two leucine-rich repeat protein-related (AT2G26380 and AT4G04220), one MLO-like protein (AT4G02600), two heat shock proteins (AT2G29970 and AT4G16660), and two dehydration-responsive proteins (AT2G34300 and AT1G 29470) (Fig. 4). Since ORS278 had no significant impact on shoot growth in the culture conditions used for our transcriptome analysis, these transcript level changes cannot be explained by an indirect effect of the PGPR strain via physiological changes. Alternatively, an appealing hypothesis would be that *Bradyrhizobium* sp. strain ORS278 might counteract some parts of defense signaling pathways of the host plant to make its colonization possible.

Role of oxylipins in ISR.

Although the leaves clearly responded to root colonization by *Bradyrhizobium* sp. strain ORS278, no major changes in the expression of defense genes have been associated with ORS278-mediated ISR, probably because this would be a metabolic burden for the host (Van Hulten et al. 2006). This observation is consistent with the study of Verhagen and coworkers (2004), which confirmed that ISR did not seem to be associated with detectable increases in expression of genes tested so far. On the other hand, our analysis and theirs underscored a set of genes whose expression was modified only after *P. syringae* pv. tomato DC3000 challenge in ISR-expressing plants and so-called primed ISR genes. Considering that genes whose expression was modified either in ISR-expressing plants or pathogen-challenging plants should not be taken into account, we found 64 primed ISR-specific genes in ORS278-colonized plants after pathogen attack as compared with the number found in control plants (Table 1). The expression of one fourth of these ISR-specific genes has been found to be modulated by JA in other studies (digital Northern performed with Genevestigator; Zimmermann et al. 2004). We focused on this subset of genes because JA has been well described as a major regulator of plant signaling involved in response to either necrotrophic pathogens, wounding, or both (Schilmiller and Howe 2005) and a key component for ISR onset (Pieterse et al. 1998). Actually, mutant analysis showed that WCS417r-mediated ISR requires an intact response to JA and ET and that this requirement is not related to any increase in the production of these hormones (Pieterse et al. 1998, 2000). Among those JA- and ET-responsive ISR-primed genes, *PDF1.2a*, a thumatin-like gene, seems of particular interest, as it is the only

ISR-primed gene shared between *Pseudomonas fluorescens* WCS417r- and *Bradyrhizobium* sp. strain ORS278-mediated ISR (Verhagen et al. 2004; this study).

Cross-talk between ET and JA signaling pathways determines the activation of a set of defense responses against pathogens and herbivores (Glazebrook 2001). A recent study identified *ERF1* as one of the molecular targets that could underlie this cross-talk (Lorenzo et al. 2003). The expression of this transcription factor could be activated synergistically by both hormones, and its ectopic expression led to the induction of the expression of pathogen-response genes. It is notable that three genes currently established to fit in the ISR-specific gene category (Verhagen et al. 2004; this study), *ASA1*, a β -1,3-glucanase (AT4G16260), and *PDF1.2a*, were induced synergistically by ET and JA and were expressed constitutively in Col-0:35S:ERF1 plants (Lorenzo et al. 2003). By contrast, the same study showed that *ASA1* was not induced by either JA or ET alone, β -1,3-glucanase was only weakly induced by ET and not by JA, and *PDF1.2a* was induced by JA but not by ET. According to these data, the former hypothesis of a signal transduction leading to ISR in which JA and ET responses are engaged successively (Pieterse et al. 1998) should be reexamined and presumably completed with a pathway in which JA and ET act synergistically (Lorenzo et al. 2003).

Although attention has focused mainly on JA as a signal, the possibility that the JA precursor 12-oxo-phytodienoic acid (OPDA) could itself be biologically active has been proposed (Mueller 1997). The isolation of an *opr3 Arabidopsis* mutant that could not produce JA but did accumulate OPDA demonstrated that OPDA was a potent regulator of defense response genes (Stintzi et al. 2001). Recently, using DNA microarrays covering 80% of the *Arabidopsis* genome, Taki and coworkers (2005) identified a group of genes that responded to OPDA but not to JA. A large percentage of OPDA-specific response genes (45%) were involved in stress responses such as wounding, salt stress, or heat shock. These included many signal transduction components and transcription factors, for instance, *WRKY53* and *ZAT10*, which were identified as ISR-specific genes in our study. The genes coding for a putative cytochrome P450 (AT5G45340) and a mitochondrial carrier (AT2G22500) protein, which have also been shown to be OPDA-specific response genes (Taki et al. 2005), appeared to be ISR-specific genes in our study (Table 1). Those four genes have never been identified as PGPR-induced nor ISR-specific in previous transcriptome large-scale analyses. Finally, 25% of the putative JA-responsive genes that we identified among ISR-specific genes appeared to actually be OPDA-responsive. To our knowledge, these figures constitute the first report of OPDA-driven expression level changes in ISR-expressing plants. This evidence suggests that OPDA may act in parallel with JA for ISR settlement. In order to assess this hypothesis, it would be helpful to determine if ISR can still be achieved, at least partly, in an *opr3* mutant of *Arabidopsis*.

Bradyrhizobium sp. strain ORS278 counterbalances the impact of *P. syringae* pv. tomato pathogenesis on host metabolism.

The MapMan overview metabolism map showing the 707 *P. syringae* pv. tomato-regulated genes identified in our study revealed a repression of photosynthetic genes involved either in light reactions or the Calvin-Benson-Bassham cycle and an induction of genes related to biotic stress (Fig. 5C). The appropriation of host resources (including sugars produced by photosynthesis) by the pathogen might have led to a concomitant regulation of photosynthesis and defense response genes. The induction of pathogenesis-related (PR) gene expression by

either exogenous sugars or ectopic expression of invertase and the characterization of the *Arabidopsis* mutant *hys1/cpr5*, which is not only hypersensitive to sugar but also constitutively expresses PR genes, form a network of evidence for the existence of a link between sugar and stress signaling (Herbers et al. 1995; Thibaud et al. 2004; Yoshida et al. 2002). It has been well-described that pathogens locally activate invertases in correlation with increased soluble sugar levels at the infection site (Chou et al. 2000). According to these data, a common sugar-sensing mechanism acting to repress photosynthetic gene expression and stimulate defense protein genes has been proposed (Sheen et al. 1999). It was actually assumed that the expression of defense-related genes was dependent on glycolysis, while sucrose exerted its control on the expression of photosynthesis genes through a glucose sensor pathway (Xiao et al. 2000).

Among the photosynthetic genes whose expression has been typically described to be mediated by a hexokinase-dependent pathway, *CAB1* (chlorophyll a/b-binding protein, AT3G54890) and *rbcS* (rubisco small subunit 3, AT5G38410) were repressed by *P. syringae* pv. tomato DC3000 (Fig. 5C; Thilmony et al. 2006; Xiao et al. 2000). Amazingly, their expression levels recovered control values when plants were 'protected' by *Bradyrhizobium* sp. strain ORS278 previous to the pathogen attack (Fig. 5D). It should be remembered here that samples used for CATMA hybridizations were collected 24 h after infection, which means prior to a detectable reduction of *Pseudomonas syringae* population in ORS278-treated plants. As a consequence, we assume that the changes observed may be directly linked to the regulatory effects of *Bradyrhizobium* sp. strain ORS278 on gene expression rather than to indirect effects of ORS278-induced ISR on pathogen population density.

A parallel pattern was observed for disease resistance proteins (AT1G52900, AT1G66090, AT1G72940, and AT5G22690) and for genes related to secondary metabolism, such as glutathione S-transferase (AT5G17220, AT1G74590, AT1G78380, or AT1G69930), whose expression levels were up-regulated by *P. syringae* pv. tomato DC3000 but resumed control levels under ISR conditions (Fig. 5A and B; Lloyd and Zakhleniuk 2004). Actually, a global analysis of the microarray data showed that, among the 707 genes exhibiting transcription changes following pathogen attack, 684 genes recovered a level of expression similar to that in control plants when the plants are protected by *Bradyrhizobium* sp. strain ORS278 colonization prior to pathogen challenge. Overall, the MapMan metabolism map demonstrated that *Bradyrhizobium* sp. strain ORS278 counteracted most of the transcription changes elicited by *P. syringae* pv. tomato DC3000 (Fig. 5). The fact that *Bradyrhizobium* sp. strain ORS278 switches off *P. syringae* pv. tomato DC3000 impact on the host transcriptome is almost certainly the mark of a successful protection. This protection could operate through priming of defense-related genes described above. Among ISR-specific genes identified in this study, a proline-rich cell wall protein (AT4G38770) seems to be a good candidate for a further reverse genetic approach, since it was down-regulated by *P. syringae* pv. tomato DC3000 and was up-regulated in ISR-expressing plants after pathogen challenge (Table 1). This peculiar pattern of expression and the property of this proline-rich protein to be rapidly insolubilized within the cell wall in response to pathogen infection strongly indicate an active role in plant defense reactions, probably through cell-wall thickening (Fowler et al. 1999).

Conclusion.

How PGPR trigger ISR remains a matter of inquiry. Gene-profiling experiments have provided a valuable tool for the characterization of a pseudomonad colonization phenotype

(Cartieaux et al. 2003; Verhagen et al. 2004; Wang et al. 2005). The original approach of Verhagen and coworkers (2004) (analysis of ISR-expressing plants challenged by pathogens) revealed the dominant role of priming for *Pseudomonas fluorescens* WCS417r-mediated ISR. Our study based on a *Bradyrhizobium* sp. strain ORS278-mediated resistance has confirmed that priming is likely to be a common feature of ISR. Furthermore, for the first time, we provide evidence that OPDA may be an additional actor in the signaling cascade leading to ISR. In addition, analyzing the microarray data with the Mapman analysis toolbox allowed us to obtain an overview of the effect of *Bradyrhizobium* sp. strain ORS278 on transcription changes induced by the pathogen. One of the most remarkable pattern thus revealed was the ability of *Bradyrhizobium* sp. strain ORS278 to counterbalance the impact of *P. syringae* pv. tomato on the host metabolism.

MATERIALS AND METHODS

Plant growth conditions.

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized by immersion in 3% (wt/vol) calcium hypochlorite and 0.01% (vol/vol) Tween 20 filtrate and were washed with sterile distilled water. The seeds were sown on a 0.8% (wt/vol) standard agar mineral medium containing 0.5 mM CaSO₄, 2 mM KNO₃, 1 mM KH₂PO₄, 0.5 mM MgCl₂, 50 μM H₃BO₃, 15 μM MnCl₂, 50 μM Na₂Fe-EDTA, 1 μM ZnCl₂, 1 μM CuCl₂, 0.03 μM (NH₄)₆Mo₇O₂₄, and 2.5 mM morpholineethanesulfonic acid (Mantelin et al. 2006). The seeds were incubated at 4°C for 48 h as dormant releasing, and then, the plates were placed in a growth chamber with 60% relative humidity at a 22°C light and 18°C dark thermoperiod with a photoperiod of 12 h of light and 12 h of dark. After a week, plants were replanted in potting soil and were maintained in the growth chamber with a photoperiod of 8 h of light and 16 h of dark. Plants were watered with tap water twice a week.

Bacteria growth conditions.

Bradyrhizobium sp. strain ORS278 was grown aerobically in yeast extract-mannitol medium (Vincent 1970) on a rotary shaker (120 rpm), with 5 days of incubation at 37°C. Bacterial cells collected by centrifugation were resuspended in sterile liquid mineral medium (Mantelin et al. 2006) to inoculate the soil to which plants were transferred to soil at a final density of 10⁷ CFU/g.

Pseudomonas syringae pv. tomato DC3000 (Whalen et al. 1991) was cultivated in King's B medium supplemented with rifampicin (50 μg/ml) at 28°C for 24 h. Cells collected by centrifugation were resuspended in 10 mM MgSO₄ to a final density of 2.5 × 10⁵ CFU/ml for manual infiltration of *Arabidopsis*.

Bioassay of ISR by *Bradyrhizobium* sp. strain ORS278.

One-week-old seedlings of *Arabidopsis* plants were transplanted into autoclaved potting soil inoculated with *Bradyrhizobium* sp. strain ORS278 in order to obtain a final concentration of bacteria of 10⁷ CFU/g. The control soil was not bacterized. To evaluate induced resistance, plants were challenge-inoculated three weeks after potting by manual infiltration of *Pseudomonas syringae* pv. tomato at a concentration of 2.5 × 10⁵ CFU/ml, and bacterial growth measurements were carried out as described by Lummerzhim and coworkers (1993).

Steady-state metabolite fingerprinting analysis using GC-EI-TOF-MS.

Soluble metabolites were extracted and analyzed essentially as described by Desbrosses and coworkers (2005). GC-EI-TOF-MS applied to the metabolite profiling of methoxyami-

nated and trimethylsilylated metabolite fractions was as first described (Wagner et al. 2003) and as detailed by Erban and associates (2007). In short, homogenization was performed in liquid nitrogen using 60 ± 5 mg of leaf material. Extraction was 300 μ l of precooled (-20°C) absolute methanol. To each sample, 30 μ l of a methylnonadecanoate stock solution (2 mg ml^{-1} methanol) (Sigma-Aldrich, Steinheim, Germany) and 30 μ l of a ribitol stock solution (0.2 mg ml^{-1} methanol) (Sigma-Aldrich) were added. After shaking at 70°C for 15 min, 200 μ l of chloroform was added to the samples, which were further shaken at 37°C for an additional 5 min. Then, 400 μ l of H_2O was added. Centrifugation was performed after vortexing at $16,000 \times g$ for 5 min at room temperature. Aliquots of the upper polar phase (80 μ l) were dried by vacuum centrifugation (Concentrator 5301, Eppendorf, Hamburg, Germany). Chemical derivatization (Fiehn et al. 2000; Roessner et al. 2000) and GC-TOF-MS metabolite profiling analysis (Erban et al. 2007; Wagner et al. 2003) was performed essentially as described previously. Retention time calibration was performed using the Kováts indices (Kováts 1958), which were calculated from standard additions to each chromatogram of a mixture of C12, C15, C19, C22, C32, C36 n-alkanes. Metabolite fingerprinting was performed after mass spectral deconvolution (ChromaTOF software version 1.00, Pegasus driver 1.61, LECO, St. Joseph, MI, U.S.A.). Peak height of each deconvoluted mass fragment representing an arbitrary mass spectral ion current was recorded and normalized using the amount of the sample fresh weight and the ion current representing the ribitol analyte for internal standardization of volume and amount variations.

Numerical and statistical analysis.

The resulting data set represents the normalized response of all deconvoluted mass fragments (N_i), which were observed in each of the biological conditions and respective replications. A log-transformed data set based on response ratios, $\log_{10}(R_i)$, was used as described earlier (Desbrosses et al. 2005). In this study, the mean normalized response value of each mass fragment was used to calculate response ratios. Missing values were substituted using replicate group averages prior to HCA, which was performed according to Desbrosses and associates (2005). ANOVA, $P < 0.05$, was used to remove all mass fragments from further analysis, which do not contribute to the differentiation of sample or which were caused by biological treatment, for example, those mass fragments that represent invariant laboratory contaminations or added internal standard compounds or metabolites that remained nonresponsive to the experimental regime. Alternatively, all mass fragments representing internal standards or laboratory contaminations were removed manually. ICA was performed after ANOVA or after manual removal of laboratory contaminations and internal standards according to Scholz and coworkers (2004, 2005).

RNA extraction and microarrays analysis.

The microarray analysis was performed using CATMA arrays containing 24,576 GST corresponding to 22,089 genes, including 21,612 AGI-predicted genes and 477 Eugene-predicted genes from *A. thaliana* (Crowe et al. 2003; Hilson et al. 2003). The GST amplicons were purified on multiscreen plates (Millipore, Bedford, MA, U.S.A.) and were resuspended in Tris-EDTA-dimethyl sulfoxide at 100 ng μl^{-1} . The purified probes were transferred to 1,536-well plates with a Genesis workstation (TECAN, Männedorf, Switzerland) and were spotted on UltraGAPS slides (Corning, NY, U.S.A.) using a Microgrid II (Genomic Solutions, Huntingdon, U.K.). The current CATMA version printed at the Unité de Recherche en Génomique Végétale (URGV, Evry, France) consists of three metablocks, each composed of 64 blocks of 144 spots. A block is a

set of spots printed with the same print-tip. In these arrays, a print-tip is used three times to print a block in each metablock.

Five comparisons were analyzed in this timecourse analysis. In the first three comparisons, each array was hybridized at the same time with cRNA from i) ORS278-protected plants, ii) *P. syringae* pv. tomato DC3000-challenged plants, and iii) ORS278/*P. syringae* pv. tomato DC3000 treated plants versus control leaves that were labeled with Cy3 and Cy5 fluorescent dyes, respectively. The fourth and fifth comparisons correspond to hybridization with cRNA from protected-challenged plants versus cRNA from ORS278-protected plants or *P. syringae* pv. tomato DC3000-challenged plants, respectively. Plants were cocultivated three weeks with *Bradyrhizobium* sp. strain ORS278 before challenge by *P. syringae* pv. tomato DC3000, and samples were collected 24 h after infection by the pathogen. This timepoint was chosen in regard to a previous study that has demonstrated that JA-responsive genes displayed a maximum of expression in ISR-expressing plants one day after infection by the pathogen (Van Wees et al. 1999). It is worthwhile to mention that 24 h postinfection neither obvious disease symptoms on infected leaves of ORS278-protected plants nor any significant difference in pathogen population size between control and ISR-expressing plants was found (data not shown). Material for each RNA extraction consists of nine independent treated leaves harvested 24 h after pathogen or MgSO_4 challenge from three plants (three leaves per plant). For each comparison, RNAs from three independent biological repetitions were pooled as recommended by Peng and coworkers (2003) in order to take into account the biological variation while minimizing the total cost. In addition, to avoid dye bias and gene-specific dye bias, a dye-swap experiment was carried out: the cRNAs labeled with Cy3 in the first array was labeled with Cy5 in the second (Martin-Magniette et al. 2005). Therefore, two arrays were used for each comparison assay.

RNA was extracted using the SV total RNA isolation system (Promega, Charbonnières, France). cRNAs were produced from 2 μg of total RNA from each pool with the Message Amp aRNA kit (Ambion, Austin, TX, U.S.A.). Then, 5 μg of cRNAs were reverse-transcribed in the presence of 200 U SuperScript II (Invitrogen), cy3-dUTP, and cy5-dUTP (NEN, Boston) for each slide, according to Puskas and coworkers (2002). Samples were combined, purified, and concentrated with YM30 Microcon columns (Millipore). Slides were prehybridized for 1 h and were hybridized overnight at 42°C in 25% formamide. Slides were washed in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) + 0.1% sodium dodecyl sulphate for 4 min, $1\times$ SSC for 4 min, $0.2\times$ SSC for 4 min, $0.05\times$ SSC for 1 min and were dried by centrifugation. A total of 20 hybridizations (10 dye-swaps) were carried out. The arrays were scanned on a GenePix 4000A scanner (Axon Instruments, Foster City, CA, U.S.A.), and images were analyzed using Genepix pro 3.0 (Axon Instruments).

Statistical analysis of microarray data experiments was designed with the statistics team of the URGV. The statistical analysis was based on one dye-swap (i.e., two arrays, each containing 24,576 GST and 384 controls). The controls were used for assessing the quality of the hybridizations but were not included in the data presented. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). No background was subtracted. In the following description, log ratio refers to the differential expression between two conditions. It is either $\log_2(\text{red/green})$ or $\log_2(\text{green/red})$ according to the experiment design. An array-by-array normalization was performed to remove systematic biases. First, we excluded spots that were considered badly formed features. We then performed a global intensity-dependent normalization using the LOESS procedure

to correct the dye bias. Finally, for each block, the log-ratio median calculated over the values for the entire block was subtracted from each individual log-ratio value to correct print-tip effects on each metablock. To determine differentially expressed genes, we performed a paired *t* test on the log ratios, assuming that the variance of the log-ratios is the same for all genes. Spots displaying extremes of variance (too small or too large) were excluded. The raw *P* values were adjusted by the Bonferroni method, which controls the family wise error rate (FWER). We considered as being differentially expressed the genes with a FWER lower than 5% (Lurin et al. 2004). The data were deposited in Array express according to the MIAME standards (accession number E-MEXP-386).

Reverse transcriptase (RT)-PCR.

Total RNA was isolated as described above. Total RNA used for RT-PCR confirmation were obtained from plants that were grown independently from those used to isolate RNA for microarray analysis. In addition, three independent biological assays were carried out from plant culture to RNA extraction. RNA quality was assayed by UV spectrophotometry. First-strand cDNA was synthesized from 500 ng of total RNA, using the SuperScript II reverse transcriptase (Invitrogen), according to manufacturer recommendations.

Real-time PCR assays were performed using the Mx3000P quantitative PCR system (Stratagene, La Jolla, CA, U.S.A.) with the qPCR mastermix for Sybr green I kit (Eurogentec, Seraing, Belgium), according to the manufacturer's recommendations. Each reaction was performed on 0.1 µl of template first cDNA strands, in a final reaction volume of 20 µl. Specific primer sets were designed for the *PAL1*, At4g16260, *AtCLH1*, *PEN3*, *ZAT10*, *ASA1*, and *PDFs* genes (Table 2). DNA amplification was performed with the following thermal cycling profile: initial denaturation at 94°C for 10 min, 40 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min), and a final extension at 72°C for 8 min. Fluorescence data were collected during the annealing stage of amplification. The presence of specific amplicon was established when fluorescence signal intensity for each molecular beacon probe exceeded the instrument-defined calculated background noise threshold level, based on the fluorescence parameters for no-template controls. The results obtained on the different treatments were standardized to the constitutive *ACT2* (actine) gene expression level.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

CATMA microarrays: www.catma.org
 Array express: www.ebi.ac.uk/arrayexpress/