Metabolic and Transcriptomic Changes Induced in Arabidopsis by the Rhizobacterium *Pseudomonas fluorescens* SS101[1][W][OA]

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Systemic resistance induced in plants by nonpathogenic rhizobacteria is typically effective against multiple pathogens. Here, we show that root-colonizing *Pseudomonas fluorescens* strain SS101 (*Pf*.SS101) enhanced resistance in Arabidopsis (*Arabidopsis thaliana*) against several bacterial pathogens, including *Pseudomonas syringae* pv *tomato* (*Pst*) and the insect pest *Spodoptera exigua*. Transcriptomic analysis and bioassays with specific Arabidopsis mutants revealed that, unlike many other rhizobacteria, the *Pf*.SS101-induced resistance response to *Pst* is dependent on salicylic acid signaling and not on jasmonic acid and ethylene signaling. Genome-wide transcriptomic and untargeted metabolomic analyses showed that in roots and leaves of Arabidopsis plants treated with *Pf*.SS101, approximately 1,910 genes and 50 metabolites were differentially regulated relative to untreated plants. Integration of both sets of "omics" data pointed to a prominent role of camalexin and glucosinolates in the *Pf*.SS101-induced resistance response. Subsequent bioassays with seven Arabidopsis mutants (*myb51*, *cyp79B2cyp79B3*, *cyp81F2*, *pen2*, *cyp71A12*, *cyp71A13*, and *myb28myb29*) disrupted in the biosynthesis pathways for these plant secondary metabolites showed that camalexin and glucosinolates are indeed required for the induction of *Pst* resistance by *Pf*. SS101. Also for the insect *S. exigua*, the indolic glucosinolates appeared to play a role in the *Pf*.SS101-induced resistance response. This study provides, to our knowledge for the first time, insight into the substantial biochemical and temporal transcriptional changes in Arabidopsis associated with the salicylic acid-dependent resistance response induced by specific rhizobacteria.

The plant microbiome harbors various fungal and bacterial genera with beneficial effects on plant growth and health (van Loon, 2007; Lugtenberg and Kamilova, 2009; Mendes et al., 2011; Pieterse et al., 2012; Zamioudis and Pieterse, 2012). Several bacterial genera, in particular *Bacillus* spp. and *Pseudomonas* spp., were shown to promote plant growth and to induce systemic resistance against fungal, oomycete, bacterial, and viral pathogens as well as insect pests (van Loon et al., 1998; Ryu et al., 2003, 2004; De Vleesschauwer and Höfte, 2009; Lugtenberg and Kamilova, 2009; Van der Ent et al., 2009; Pineda et al., 2010). Well-studied examples of induced resistance include systemic acquired resistance (SAR) and induced systemic resistance (ISR; Choi et al., 2011; Pieterse et al., 2012). Both SAR and ISR function systemically throughout the plant (Conrath et al., 2002). While SAR is triggered by necrotizing pathogens (Conrath et al., 2002), ISR is activated by selected nonpathogenic plant growth-promoting rhizobacteria (PGPR; Van der Ent et al., 2009). The systemic resistance response induced in plants by these nonpathogenic rhizobacteria is in many cases regulated by the phytohormones jasmonic acid (JA) and ethylene (ET; van Loon et al., 1998; van Hulten et al., 2006; Pozo et al., 2008; Van der Ent et al., 2009; Zamioudis and Pieterse, 2012) and associated with the expression of *PDF1.2*, the gene encoding plant defensin1.2 (Van Oosten et al., 2008). Several PGPR trigger systemic resistance by priming the plant for activation of various cellular defense responses that are subsequently induced upon pathogen attack (Conrath, 2006). The potentiated responses include oxidative burst (Iriti et al., 2003), cell wall reinforcement (Benhamou and Bélanger, 1998), and the production of secondary metabolites (Yedidia et al., 2003). However, some rhizobacteria do not induce systemic resistance via the JA/ET pathway but via the salicylic acid (SA) pathway (Maurhofer et al., 1994, 1998; De Meyer and Höfte, 1997; De Meyer et al., 1999; Audenaert et al., 2002; Barriuso et al., 2008). The SA-dependent signaling pathway results in local and systemic increases in endogenously synthesized SA, leading to activation of the regulatory protein *nonexpressor*

[1] This work was supported by the Dutch Technology Foundation and by the Netherlands Genomics Initiative ECOLINC program.

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www.plantphysiol.org/cgi/doi/10.1104/pp.112.207324

of pathogenesis-related (*PR*) *genes1* (*NPR1*) and subsequent expression of genes encoding PR proteins, including PR1, PR2, and PR5 (Ward et al., 1991; van Loon and Van Strien, 1999).

To date, there is little information available on metabolic changes induced in plants by PGPR that enhance resistance against pathogens or insect pests (Walker et al., 2011; Weston et al., 2012). Furthermore, for only few rhizobacterial strains, transcriptomic analyses have been conducted to unravel the molecular changes in plants associated with induced systemic resistance (Cartieaux et al., 2003, 2008; Verhagen et al., 2004; Wang et al., 2005; Weston et al., 2012). Almost all of these microarray studies revealed relatively few transcriptional changes: for instance, plant-growth promoting *Pseudomonas thivervalensis* strain MLG45 induced transcriptional changes in 63 genes in the shoots and in nine genes in the roots of Arabidopsis (*Arabidopsis thaliana*), with an increase in defense-related transcripts and a repression of photosynthesis-related genes (Cartieaux et al., 2003). In the transcriptome analysis by Verhagen et al. (2004), 97 genes were differentially regulated in the roots upon colonization by *Pseudomonas fluorescens* strain WCS417r (*Pf*.WCS417r), but no detectable transcriptional changes were found in the shoots. It should be noted that these two bacterial strains induce systemic resistance in Arabidopsis via the JA/ET pathway. In contrast, genome-wide transcriptional changes induced by rhizobacteria that trigger resistance via the SA pathway have, to our knowledge, not been reported yet.

In this study, transcriptomic and untargeted metabolomic approaches were applied and integrated to elucidate the molecular and biochemical changes induced in Arabidopsis by the nonpathogenic rhizobacterium *Pseudomonas fluorescens* SS101 (*Pf*.SS101). *Pf*. SS101 was originally isolated from the wheat (*Triticum aestivum*) rhizosphere (De Souza et al., 2003) and produces massetolide A, a cyclic lipopeptide surfactant with diverse natural functions, including a role in swarming motility, biofilm formation, and defense against protozoan predators (de Bruijn et al., 2008; Mazzola et al., 2009; Raaijmakers et al., 2010). Massetolide A has destructive effects on zoospores of multiple oomycete plant pathogens (De Souza et al., 2003; de Bruijn et al., 2008; van de Mortel et al., 2009) and induces systemic resistance in tomato (*Solanum lycopersicum*) leaves against *Phytophthora infestans* (Tran et al., 2007). Here, we show that the resistance response induced by *Pf*.SS101 in Arabidopsis against the bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) is mediated by SA signaling and NPR1 but not by JA/ET signaling. Integration of the plant transcriptome and metabolome data pointed to a prominent role of camalexin and glucosinolates in the *Pf*.SS101-induced resistance response. The role of these metabolites in ISR was tested in bioassays with Arabidopsis mutants disrupted in the biosynthesis pathways for these plant secondary metabolites.

RESULTS

Pf.SS101 Induces Resistance in Arabidopsis against Diverse Pathogenic Bacteria and the Herbivore Insect *Spodoptera exigua*

Soil bioassays showed that treatment of seeds or root tips of Arabidopsis with *Pf*.SS101 significantly reduced disease incidence caused by *Pst* by approximately 10% to 30% after 18 d of growth (Fig. 1A, a–c). Significant reductions in *Pst* disease incidence were also observed for *Pf*.SS101-treated Arabidopsis seedlings grown in vitro on vertically positioned one-half-strength Murashige and Skoog agar plates (Fig. 1A, d–f). Consistent with these macroscopic observations, the population density of *Pst* in Arabidopsis leaves was reduced in plants treated with *Pf*.SS101 (Fig. 1A). In the soil bioassays with *Pf*.SS101-treated seeds, *Pf*.SS101 established population densities on the roots and leaves of Arabidopsis of 1×10^6 colony-forming units (CFU) mg^{-1} and 5×10^5 CFU mg^{-1}, respectively, after 18 d of plant growth. Root tip inoculation of 7-d-old seedlings with *Pf*.SS101 resulted in a population density of *Pf*.SS101, after 18 d of plant growth, of 1×10^6 CFU mg^{-1} on the roots, while on the shoot, *Pf*.SS101 was not detectable or was found at densities close to the detection limit of 1×10^4 CFU mg^{-1} leaf tissue. In vitro bioassays with a *GFP*-tagged derivative of *Pf*.SS101 further showed that, at 18 d after root tip treatment of Arabidopsis seedlings, *Pf*.SS101 was only occasionally found on cotyledons but not on true leaves. These data indicate that after root tip inoculation, *Pf*.SS101 and *Pst* are physically separated. In the root tip inoculation experiments, the reduction in disease incidence in the *Pf*.SS101 treatment did not coincide with a statistically significant reduction in *Pst* density, which seems to be due to relatively large variations in *Pst* densities among the biological replicates (Fig. 1A, e).

The bioassays further showed that strain *Pf*.SS101 suppressed diseases in Arabidopsis caused by various other bacterial pathogens, including *Pseudomonas syringae* pv *maculicola*, *Pseudomonas alisalensis*, and *Pseudomonas viridiflava* (Fig. 1B). For these three bacterial pathogens, disease incidence was substantially lower than that of *Pst* in spite of the fact that the population densities of *P. alisalensis* and *P. viridiflava* were comparable to *Pst*. This suggests that these two bacterial strains are less virulent on Arabidopsis than *Pst*. *P. syringae* pv *maculicola* established a population density of 1.1×10^6 CFU mg^{-1}, which was approximately 6 times less than that of *Pst* (6.3×10^6 CFU mg^{-1}) and which may have contributed to the lower disease incidence (Fig. 1B). Larval mortality of the insect herbivore *S. exigua* was significantly higher when feeding on *Pf*.SS101-treated Arabidopsis plants as compared with untreated plants (Fig. 1C).

Pf.SS101-Mediated Systemic Resistance Is Dependent on SA Signaling and NPR1

To unravel the signal transduction pathways involved in the resistance response induced by *Pf*.SS101

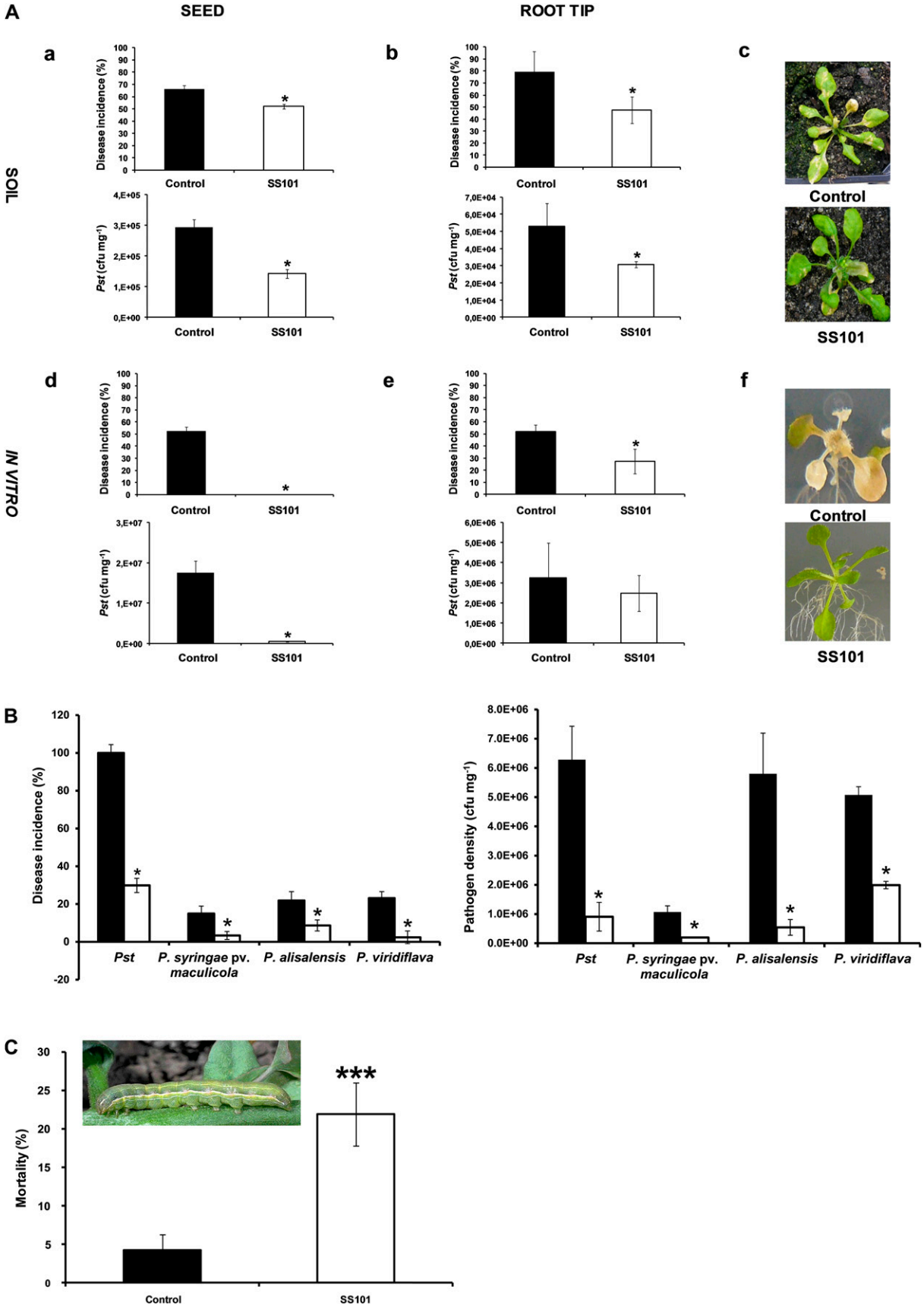

Figure 1. Effects of seed or root tip treatment of Arabidopsis wild-type Col-0 with *Pf*.SS101 on resistance against different bacterial pathogens and the lepidopteran insect *S. exigua*. A, Disease incidence and *Pst* population density on leaves of

against *Pst*, Arabidopsis mutants of the JA, ET, or SA signaling pathways were tested. In soil bioassays, root tip treatment with *Pf*.SS101 led to a significant ($P < 0.05$) reduction in disease incidence in both wild-type Columbia (Col-0) and in the *ethylene insensitive2* (*ein2*) and *jasmonate resistance1* (*jar1*) mutants but not in the *nahG* and *npr1-1* mutants (Fig. 2A). The rhizosphere population densities of *Pf*.SS101 after 18 d of plant growth were comparable for the different Arabidopsis mutants: on average, 2×10^7 CFU mg^{-1} for Col-0 and *npr1-1* and 4 to 5×10^7 CFU mg^{-1} for *ein2*, *jar1*, and *nahG*. For almost all of the Arabidopsis mutants, similar effects on *Pst* disease incidence were obtained in the in vitro plate assays (Fig. 2B). An exception was the response of the *npr1-1* mutant, which showed an increased disease incidence when treated with *Pf*.SS101 (Fig. 2B). In these in vitro assays, we also included mutant *npr1-3*. Compared with *npr1-1*, which is impaired in JA/ET/SA signaling, *npr1-3* is only impaired in SA signaling (Cao et al., 1994, 1997). These results showed that also in mutant *npr1-3*, *Pf*.SS101 did not induce resistance against *Pst* (Fig. 2B). Collectively, these results suggest that *Pst* resistance induced in Arabidopsis by *Pf*.SS101 is dependent on SA signaling and NPR1 rather than on JA and ET signaling.

To further support these findings, we tested Arabidopsis mutants *myb72* and *myc2* (Fig. 2C) and also determined the SA concentrations in roots and leaves of Arabidopsis plants treated with *Pf*.SS101 (Fig. 2D). The MYB domain protein72 (MYB72) is an essential signaling compound for ISR by *Pf*.WCS417r, and also mutants impaired in the JASMONATE-INSENSITIVE1/MYC2 gene (*jin1-1* and *jin1-2*) were unable to mount *Pf*. WCS417-ISR against *Pst* and the downy mildew pathogen *Hyaloperonospora parasitica* (Pozo et al., 2008; Van der Ent et al., 2008). The results of our experiments showed that *Pf*.SS101 significantly reduced disease incidence in both the *myb72* and *myc2* mutants (Fig. 2C). The *coronatine insensitive1-16* (*coi1-16*) mutant, included in these assays as a control, showed a low disease incidence (Fig. 2C), which is consistent with previous findings (Feys et al., 1994; Kloek et al., 2001). Combined with the significantly higher SA (free and bound) concentrations detected in roots of Arabidopsis plants treated with *Pf*.SS101 (Fig. 2D), we conclude that *Pst* resistance induced in Arabidopsis by *Pf*.SS101 is dependent on SA signaling and NPR1 and not on JA and ET signaling. These experiments also showed that the results of the in vitro assays (Fig. 2B) were consistent with those obtained in the soil bioassays (Fig. 2A). Hence, we decided to use the in vitro assays to unravel the transcriptomic and metabolomic changes induced in Arabidopsis by *Pf*. SS101.

Genome-Wide Transcriptional Changes Induced in Arabidopsis by *Pf*.SS101

Microarray analyses were performed at 10, 14, and 18 d of plant growth to monitor transcriptional changes induced in Arabidopsis by *Pf*.SS101. Seed treatment with *Pf*.SS101 resulted in a total of 1,179 and 920 differentially expressed genes (2-fold or greater; false discovery rate ≤ 0.05) in Arabidopsis roots and leaves, respectively (Fig. 3A). In roots, 556 genes were induced and 623 genes were repressed, while in leaves, the majority of the differentially expressed genes (687) were up-regulated (Fig. 3A). A total of 418 genes in roots and 343 genes in leaves were differentially expressed only at 10 d post inoculation (DPI; Fig. 3, B and C). Although the largest number of differentials was found at 10 DPI, other differentially regulated genes were found at 14 and 18 DPI (Supplemental Tables S1 and S2).

Overrepresentation analysis (ORA) of the 556 genes up-regulated in the roots resulted in 66 functional categories, with a large group of genes involved in defense, stress response, root morphogenesis, metal ion transport, secondary metabolism, and SA signaling (Supplemental Table S3). With regard to genes involved in SA signaling, five genes (*PBS3*, *WRKY60*, *WRKY70*, *AtMYB45*, and *ATEXT4*) were significantly up-regulated in the roots (Table I). Several genes involved in glucosinolate biosynthesis were overrepresented among the genes that were differentially regulated in the roots (Table I); these include *MAM1*, *BCAT4*, and *CYP83A1*, which are genes involved in the biosynthesis of aliphatic glucosinolates (Yan and Chen, 2007; Albinsky et al., 2010; Sønderby et al., 2010). The 623 genes that were down-regulated in roots were divided into 38 functional categories, representing genes involved in responses to hormones and different abiotic stress factors such as temperature and reactive oxygen species (Supplemental Table S4).

Figure 1. (*Continued.*)

Arabidopsis grown in soil or in vitro. *Pst* was applied 18 d (for soil assays; a–c) or 14 d (for in vitro assays; d–f) after seed or root tip treatment with *Pf*.SS101. Symptoms were determined 5 to 7 d after inoculation. Values shown are means ± SE of 20 to 30 plants. Experiments were performed at least twice, and representative results are shown. Asterisks indicate statistically significant differences from the control treatment (one-way ANOVA, $P < 0.05$). B, Disease incidence of Arabidopsis leaves after inoculation with *Pst*, *P. syringae* pv *maculicola*, *P. alisalensis*, or *P. viridiflava*. Arabidopsis Col-0 seeds were treated with *Pf*.SS101 and grown under in vitro conditions. Symptoms and population densities of these pathogens were determined 5 d after inoculation. Values shown are means ± SE of four independent replicates of 10 plants each. Experiments were performed at least twice, and representative results are shown. Asterisks indicate statistically significant differences from the control treatment (one-way ANOVA, $P < 0.05$). C, Effects of *Pf*.SS101 on larval mortality of the lepidopteran insect *S. exigua* after 6 d of feeding. *Pf*.SS101 was inoculated on root tips of Arabidopsis seedlings grown in vitro. Values shown are back-transformed mortality means ± SE based on 45 to 48 plates per treatment (two-way GLM). Experiments were performed twice with similar results. Asterisks indicate statistically significant differences from the control treatment (***$P < 0.001$). The inset shows a photograph of *S. exigua* (courtesy of Tibor Bukovinszky; www.bugsinthepicture.com).

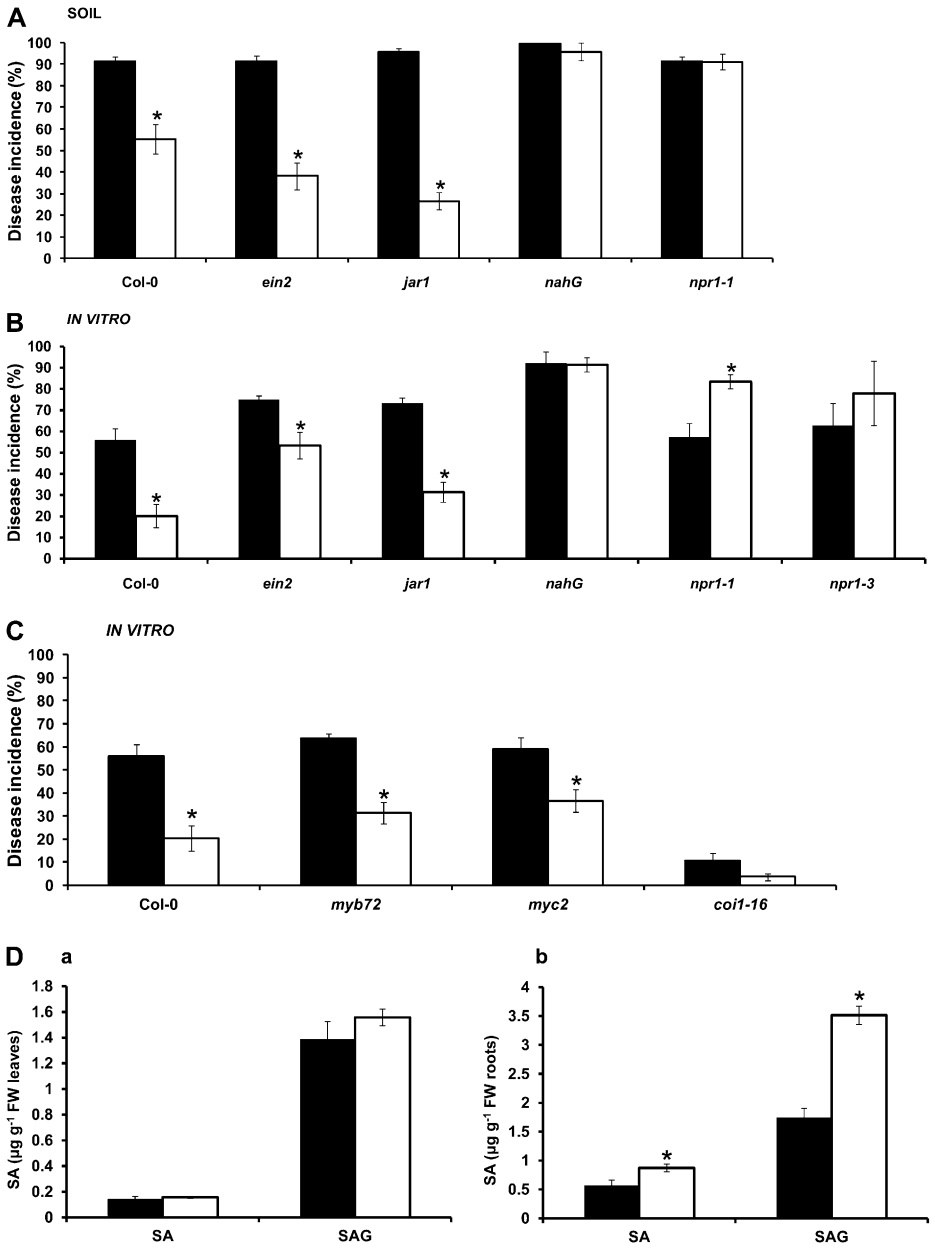

Figure 2. *Pf*.SS101 treatment induces resistance in Arabidopsis independent of JA and ET signaling but dependent on SA signaling. A, Disease incidence of *Pst* on Arabidopsis wild type (Col-0) and various mutants grown in soil; the mutants tested were JA (*jar1*), ET (*ein2*), and SA (*nahG*, *npr1*) signaling mutants. Values shown are means ± SE of 20 to 30 plants. Experiments were repeated twice with similar results. Symptoms were determined 5 to 7 d after inoculation. B and C, Disease incidence of *Pst* on Arabidopsis wild type (Col-0), JA/ET (*ein2*, *jar1*, *myb72*, *myc2*), and SA (*nahG*, *npr1*) signaling mutants grown under in vitro conditions. Symptoms were determined 5 to 7 d after inoculation. D, Accumulation of free SA and SA-2-*O*-β-D-glucoside (SAG) in Arabidopsis leaves (a) and roots (b). Values shown are means ± SE of four independent replicates of 10 plants each. Black bars represent mock-inoculated plants (control), and white bars represent *Pf*.SS101 root tip-inoculated plants. Asterisks indicate statistically significant differences from the control treatment (one-way ANOVA, $P < 0.05$). FW, Fresh weight.

The 687 genes that were up-regulated in the leaves were divided into 142 functional categories (Supplemental Table S5). A large group of these genes are involved in defense, secondary metabolism, and responses to different stimuli. At least 21 genes involved in SA signaling were significantly up-regulated in the leaves, with the PR1 gene more than 185-fold at 10 DPI (Fig. 4A; Table I; Supplemental Table S5). The observed transcriptional changes induced by *Pf*.SS101 support the results from the bioassays that the SA pathway plays an important role in the *Pf*.SS101-induced resistance response (Figs. 2 and 4A). Other genes that were overrepresented among the up-regulated genes in the leaves included genes involved in the phenylpropanoid, flavonoid, glucosinolate, and camalexin biosynthesis pathways (Table I; Supplemental Table S5). The 233 genes that were down-regulated in the leaves by *Pf*.SS101 were divided into 27 functional categories and represent genes involved in development and responses to diverse abiotic stimuli such as radiation, cold, and desiccation (Supplemental Table S6).

Metabolic Changes Induced in Arabidopsis by *Pf*.SS101

To get more insight into the effects of *Pf*.SS101 on the metabolism of Arabidopsis, specifically its secondary metabolite profile, we applied an untargeted reverse-phase liquid chromatography-mass spectrometry (LC-MS)-based metabolomics approach of aqueous

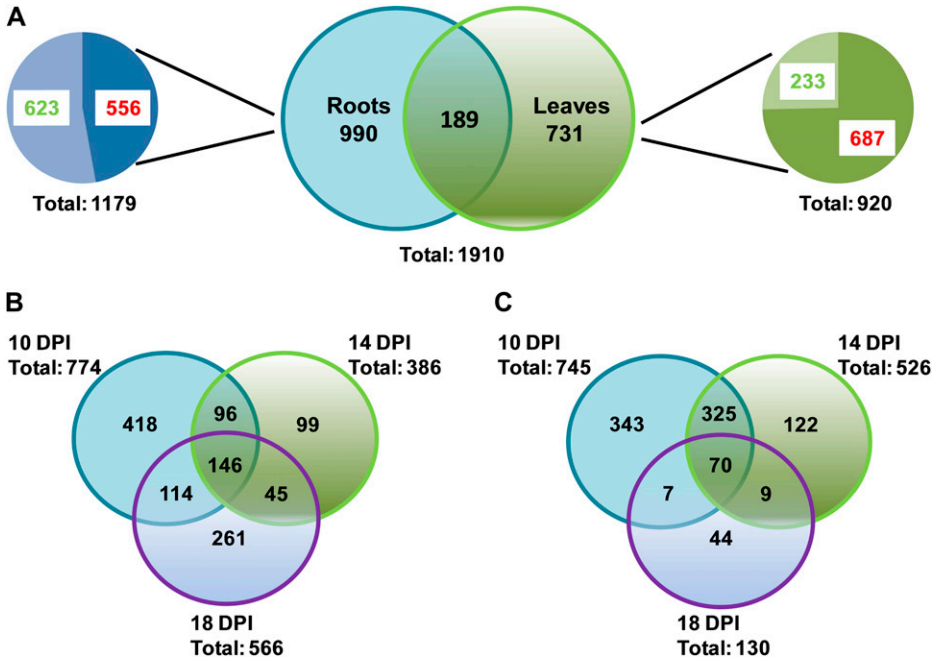

Figure 3. Genome-wide transcriptional changes induced in Arabidopsis by *Pf*.SS101. A, Total number of induced (red) and repressed (green) genes in roots or in leaves of Arabidopsis treated with *Pf*.SS101. B and C, Venn diagrams displaying the overlap in differentially regulated Arabidopsis genes in roots (B) or in leaves (C) at 10, 14, and 18 DPI with *Pf*.SS101.

methanol extracts from 18-d-old plants treated with *Pf*.SS101 or not treated (controls). This platform enabled the comparison of samples for the relative abundance of numerous metabolites, including major Arabidopsis secondary metabolites like glucosinolates, phenylpropanoids, and flavonoids.

To identify significantly altered metabolites, 931 mass features with a signal-to-noise ratio of 10 or higher were extracted from the raw LC-MS data files in an unbiased manner, using Metalign software, and individually compared between *Pf*.SS101-treated and nontreated Arabidopsis by Student's *t* test. Each mass signal was assigned to one of the 129 reconstructed metabolites, as determined by MSClust software. A total of 198 mass signals, representing 46 different metabolites, were significantly changed in roots of *Pf*.SS101-treated plants as compared with nontreated plants (Fig. 5; Supplemental Table S7). In leaves, significant effects of *Pf*.SS101 were found for 28 mass signals representing 13 metabolites (Fig. 5; Supplemental Table S7). For the 46 differentials in the roots, 31 metabolites were significantly increased relative to the nontreated plants (ratio ≥ 2, $P < 0.01$) and 15 metabolites were significantly decreased (ratio ≤ 0.5, $P < 0.01$; Supplemental Table S7). The indolyl glucosinolates glucobrassicin (indol-3-ylmethyl glucosinolate) and 1- or 4-methoxyglucobrassicin (1- or 4-methoxy-indol-3-ylmethyl) as well as the long-chain aliphatic glucosinolates glucohesperin, glucoibarin [7-(methylsulfinyl)heptyl glucosinolate], 7-(methylthio)heptyl glucosinolate, glucohirsutin (8-methylsulfinyloctyl glucosinolate), and 8-methylthiooctyl glucosinolate were all significantly increased in roots of *Pf*.SS101-treated plants (Fig. 5A; Supplemental Table S7). Besides these changes in glucosinolates, significant increases in the coumarin phytoalexin scopoletin glucoside, D-gluconate, and indole-3-carboxylic acid β-D-glucopyranosyl ester were detected (Fig. 5A; Supplemental Table S7). Significant decreases were observed for Cys, sinapoyl malate, and quercetin 7-*O*-rhamnoside 3-*O*-rhamnosylglucoside (Fig. 5B; Supplemental Table S7).

In the leaves, 11 metabolites were significantly increased and two metabolites were found at significantly lower levels in the *Pf*.SS101-treated seedlings (Supplemental Table S7). Also in the leaves, the glucosinolates were among the most differentially accumulating metabolites. The long-chain methylsulfinylalkyl glucosinolates glucohesperin, glucoibarin, and glucohirsutin, as well as D-gluconate and indole-3-carboxylic acid β-D-glucopyranosyl ester, were increased 2- to 7-fold in the leaves (Fig. 5C; Supplemental Table S7). Collectively, these results indicated that *Pf*.SS101 stimulated the biosynthesis of indolyl and aliphatic glucosinolates, scopoletin glucoside, D-gluconate, and indole-3-carboxylic acid β-D-glucopyranosyl ester. *Pf*.SS101 also induced the biosynthesis of various other, yet unknown, metabolites not found previously in other documented metabolomics studies with Arabidopsis (Tohge et al., 2005; Malitsky et al., 2008; Albinsky et al., 2010; Supplemental Table S7).

Pf.SS101-Induced Resistance Is Dependent on Glucosinolates and Camalexin

Both the metabolome and transcriptome analyses of *Pf*.SS101-treated Arabidopsis plants revealed substantial changes in the leaves for the biosynthesis pathways of

Table I. *Differentially expressed genes in Arabidopsis treated with Pf.SS101 involved in SA signaling, glucosinolate, and camalexin biosynthesis*
Plants were grown for 10, 14, or 18 d. Boldface values indicate the genes that were significantly differentially expressed. Contr., control.

Arabidopsis Genome Initiative Code	Name	Description	SS101/ Contr. Roots 10 d[a]	SS101/ Contr. Roots 14 d[a]	SS101/ Contr. Roots 18 d[a]	SS101/ Contr. Leaves 10 d[a]	SS101/ Contr. Leaves 14 d[a]	SS101/ Contr. Leaves 18 d[a]
SA-mediated signaling								
AT1G21250	WAK1	Cell wall-associated kinase	1.19	1.17	1.08	**8.28**	**6.37**	1.67
AT1G21270	WAK2	Cytoplasmic Ser/Thr protein kinase	1.10	−1.00	1.05	**2.96**	2.41	2.01
AT1G64280	NPR1	Key regulator of the SA-mediated SAR pathway	1.46	1.22	1.07	1.76	1.73	1.41
AT1G74710	ICS1/SID2	Encodes a protein with isochorismate synthase activity	1.23	1.32	1.33	**2.58**	1.62	−1.20
AT1G75040	PR5	Thaumatin-like protein involved in response to pathogens	1.49	1.16	−1.12	**2.31**	1.60	−1.17
AT1G76930	ATEXT4	Encodes an Arabidopsis extensin gene	1.63	1.51	**2.40**	**7.00**	**3.46**	1.24
AT1G80840	WRKY40	Pathogen-induced transcription factor	3.89	1.79	−1.03	**5.87**	1.85	2.15
AT2G14610	PR1	PR1 gene expression is induced in response to a variety of pathogens	1.02	1.02	1.09	**186.36**	**61.91**	4.59
AT2G25000	WRKY60	Pathogen-induced transcription factor	−1.07	1.33	**2.25**	**5.81**	**3.86**	1.12
AT3G11820	SYP121	Encodes plasma membrane syntaxin	1.02	−1.16	−1.48	**2.11**	**2.13**	1.56
AT3G48090	EDS1	Component of R gene-mediated disease resistance	1.52	1.04	−1.01	**2.49**	2.11	1.03
AT3G48920	ATMYB45	Member of the R2R3 factor gene family	1.97	1.89	**2.08**	4.55	1.72	1.40
AT3G52400	SYP122	Syntaxin protein	−1.80	−1.21	−1.27	**3.91**	**3.85**	**2.39**
AT3G52430	PAD4	Encodes a lipase-like gene protein	1.84	1.39	1.49	**3.94**	**3.37**	1.11
AT3G56400	WRKY70	Member of WRKY transcription factor; group III	**2.80**	−1.39	−1.68	**4.13**	**3.25**	2.61
AT3G61190	BAP1	Encodes a protein with a C2 domain that binds to BON1	−1.51	−1.54	−1.76	**3.37**	3.01	−1.19
AT4G14400	ACD6	Encodes a novel protein with putative ankyrin and transmembrane regions	1.37	1.19	−1.08	**4.47**	2.85	1.82
AT4G31800	WRKY18	Pathogen-induced transcription factor	1.30	1.03	−1.28	**10.49**	**9.84**	3.37
AT4G39030	EDS5/SID1	Encodes an orphan multidrug and toxin extrusion transporter	−1.05	1.11	1.58	**5.18**	**3.73**	−1.29
AT5G13320	PBS3	Signaling gene in disease resistance	**2.04**	1.84	**2.17**	**3.24**	**2.17**	−1.33
AT5G40990	GLIP1	Component of plant resistance	1.21	1.29	1.46	**3.17**	**2.51**	1.59
AT5G54610	ANK	Belongs to the ankyrin repeat protein family	1.10	1.04	1.25	**28.84**	**37.00**	**4.06**
Indole glucosinolate biosynthesis pathway								
AT1G18570	MYB51	R2R3-MYB transcription family	1.67	1.50	1.22	**7.29**	**4.82**	**2.99**
AT1G21100	IGMT1	*O*-Methyltransferase, putative	**−2.88**	−1.53	−1.39	**4.95**	**2.80**	−1.30
AT1G21120	IGMT2	*O*-Methyltransferase, putative	**6.21**	2.76	**4.07**	**9.01**	**3.52**	1.92
AT1G24100	UGT74B1	Encodes a UDP-Glc:thiohydroximate *S*-glucosyltransferase	1.27	1.43	1.54	1.04	1.17	−1.01
AT1G74080	MYB122	R2R3-MYB transcription family	1.53	1.77	1.62	1.81	1.38	1.01
AT1G74100	AtSOT16/ AtSTa	Sulfotransferase family protein	1.06	1.01	1.15	1.63	1.63	1.11
AT1G74100	AtST5a	Sulfotransferase family protein	1.06	1.01	1.15	1.63	1.63	1.11
AT2G20610	SUR1/C-S lyase	Confers auxin overproduction; mutants have an overproliferation of lateral roots	1.10	1.10	1.39	1.09	1.43	−1.07
AT2G22330	CYP79B3	Encodes cytochrome P450 CYP79B3	−1.28	1.27	1.25	**2.04**	1.28	1.38
AT2G30860	ATGSTF9	Encodes glutathione transferase belonging to the ϕ class	1.02	1.04	1.11	1.22	1.28	1.07
AT2G30870	ATGSTF10	Encodes glutathione transferase belonging to the ϕ class	1.04	1.02	1.03	1.37	1.43	1.18
AT2G44490	AtPEN2	β-Glucosidase26	1.02	−1.06	−1.15	−1.12	−1.18	−1.07
AT4G24520	MYB34/ ATR1	CYP450 reductase likely to be involved in phenylpropanoid metabolism	−1.28	−1.12	−1.26	−1.06	−1.19	1.14
AT4G30530	GGP1	Defense-related protein, putative	1.26	1.28	1.46	1.23	1.24	1.16

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Arabidopsis Genome Initiative Code	Name	Description	SS101/ Contr. Roots 10 d[a]	SS101/ Contr. Roots 14 d[a]	SS101/ Contr. Roots 18 d[a]	SS101/ Contr. Leaves 10 d[a]	SS101/ Contr. Leaves 14 d[a]	SS101/ Contr. Leaves 18 d[a]
AT4G31500	CYP83B1/ SUR2	Encodes an oxime-metabolizing enzyme in the biosynthetic pathway of glucosinolates	−1.02	1.23	1.54	**2.10**	1.67	1.18
AT4G31970	CYP82C2	Member of CYP82C	1.31	1.17	1.15	**86.60**	**62.03**	**29.45**
AT4G37400	CYP81F3	Member of CYP81F	1.07	1.61	1.88	1.06	1.10	1.01
AT4G37410	CYP81F4	Member of CYP81F	−1.28	−1.03	−1.12	−1.07	−1.07	1.43
AT4G37430	CYP81F1	Member of CYP81F	1.26	−1.05	1.16	1.15	1.19	−1.07
AT4G39950	CYP79B2	Encodes cytochrome P450 CYP79B2	−1.04	1.30	1.79	**3.21**	**2.68**	1.66
AT5G05260	CYP79A2	Encodes cytochrome P450 CYP79A2	1.07	1.12	−1.01	−1.08	−1.06	−1.37
AT5G05730	ASA1	α-Subunit of anthranilate synthase	1.71	1.68	**2.53**	**2.27**	1.77	1.26
AT5G57220	CYP81F2	Member of CYP81F	**3.74**	**3.04**	**2.31**	**17.64**	**7.94**	2.97
Camalexin biosynthesis pathway								
AT2G30750	CYP71A12	Putative cytochrome P450	**25.56**	**5.87**	**10.43**	**83.61**	**40.44**	**13.02**
AT2G30770	CYP71A13	Putative cytochrome P450	1.86	1.90	1.48	**5.30**	**5.59**	2.02
AT3G26830	CYP71B15/ PAD3	Encodes a cytochrome P450 enzyme	1.05	−1.47	1.02	**14.53**	**9.09**	1.92
Aliphatic glucosinolate biosynthesis pathway								
AT1G10060	BCAT1	Encodes a mitochondrial branched-chain amino acid aminotransferase	−1.06	−1.05	1.13	**-2.38**	−1.50	−1.59
AT1G10070	BCAT2	Encodes a chloroplast branched-chain amino acid aminotransferase	−1.26	−1.79	−1.59	1.13	1.34	**-3.13**
AT1G12140	At-GS-OX5	Flavin-containing monooxygenase amily protein/FMO family protein	−1.26	1.00	1.07	1.18	−1.03	−1.22
AT1G18590	AtSOT17	Sulfotransferase family protein	1.00	1.30	**2.19**	−1.05	1.41	1.47
AT1G62540	At-GS-OX2	Flavin-containing monooxygenase family protein/FMO family protein	−1.76	−1.56	−1.16	−1.12	−1.13	1.28
AT1G62560	At-GS-OX3	Flavin-containing monooxygenase family protein/FMO family protein	**2.33**	1.84	**2.87**	−1.15	1.31	1.21
AT1G62570	At-GS-OX4	Flavin-containing monooxygenase family protein/FMO family protein	1.23	1.15	1.14	−1.26	−1.07	1.32
AT1G65860	At-GS-OX1	Flavin-containing monooxygenase family protein/FMO family protein	1.35	1.25	1.02	−1.01	1.25	−1.03
AT1G74090	AtSOT18	Sulfotransferase family protein	1.10	1.17	1.53	−1.07	1.23	1.09
AT1G78370	ATGSTU20	Encodes glutathione transferase belonging to the τ class	−1.31	−1.00	**3.19**	−1.19	1.28	1.43
AT2G20610	SUR1	Confers auxin overproduction; mutants have an overproliferation of lateral roots	1.10	1.10	1.39	1.09	1.43	−1.07
AT2G31790	UGT74C1	UDP-glucoronosyl/ UDP-glucosyltransferase family protein	1.39	1.69	**2.60**	1.08	1.16	1.22
AT2G43100	IPMI SSU2	Aconitase C-terminal domain-containing protein	1.17	1.25	**2.56**	1.01	1.27	1.26
AT3G03190	ATGSTF11	Encodes glutathione transferase belonging to the ϕ class	**2.56**	**3.46**	**5.83**	−1.16	1.27	2.12
AT3G19710	BCAT4	Belongs to the branched-chain amino acid aminotransferase gene family	1.87	**2.28**	**7.30**	−1.03	1.44	1.27
AT3G49680	BCAT3	Encodes a chloroplast branched-chain amino acid aminotransferase	1.04	1.05	1.24	1.00	1.16	−1.03
AT3G58990	IPMI SSU3	Aconitase C-terminal domain-containing protein	1.41	1.86	**3.50**	−1.01	1.44	1.53
AT4G03050	AOP3	Encodes a 2-oxoglutarate-dependent dioxygenase	−1.08	−1.10	−1.07	1.13	−1.25	1.01
AT4G03060	AOP2	Encodes a 2-oxoglutarate-dependent dioxygenase	−1.02	1.31	1.12	−1.94	1.55	1.10
AT4G03070	AOP1	Encodes a possible 2-oxoglutarate-dependent dioxygenase	1.34	1.61	1.76	−1.50	1.06	−1.26

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Arabidopsis Genome Initiative Code	Name	Description	SS101/ Contr. Roots 10 d[a]	SS101/ Contr. Roots 14 d[a]	SS101/ Contr. Roots 18 d[a]	SS101/ Contr. Leaves 10 d[a]	SS101/ Contr. Leaves 14 d[a]	SS101/ Contr. Leaves 18 d[a]
AT4G12030	BAT5/ BASS5	Bile acid:sodium symporter family protein	1.45	1.17	**2.65**	−1.01	1.11	1.63
AT4G13430	IPMI LSU1	Aconitase family protein	1.23	1.12	1.58	1.07	1.07	−1.01
AT4G13770	CYP83A1/ REF2	Putative cytochrome P450	1.70	**2.08**	**5.85**	1.00	1.08	1.25
AT4G30530	GGP1	Defense-related protein, putative	1.26	1.28	1.46	1.23	1.24	1.16
AT5G07690	MYB29	Encodes a putative transcription factor	1.02	1.16	−1.12	−1.38	1.41	1.58
AT5G07700	MYB76	Encodes a putative transcription factor	1.06	1.06	1.16	1.01	1.07	−1.00
AT5G23010	MAM1	Encodes a methylthioalkylmalate synthase	**2.18**	**2.50**	**6.04**	1.04	1.24	1.16
AT5G23020	MAM-L/ MAM3	Methylthioalkymalate synthase-like	1.31	1.44	**2.26**	**2.55**	2.07	4.11
AT5G61420	MYB28	Encodes a putative transcription factor	1.06	1.22	2.55	−1.08	−1.28	1.24

[a]Ratio of significant (false discovery rate < 0.05) differentially (two or more) expressed genes between *Pf*.SS101-treated plants and control (mock-treated) plants.

indolyl glucosinolates and camalexin (Figs. 4B and 5; Supplemental Table S7). Transcript levels of genes involved in aliphatic glucosinolate biosynthesis were also significantly increased in roots of *Pf*.SS101-treated Arabidopsis seedlings at 18 DPI (Fig. 4C). Quantitative PCR was performed to confirm the microarray data and to measure the expression level of *CYP79F1* and *CYP79F2*, two genes involved in aliphatic glucosinolate biosynthesis but not represented on the Affymetrix ATH1 microarray. The results confirmed that these two genes and other genes involved in indolic and aliphatic glucosinolate biosynthesis as well as camalexin biosynthesis were up-regulated in roots and leaves of *Pf*.SS101-treated plants (Supplemental Table S8).

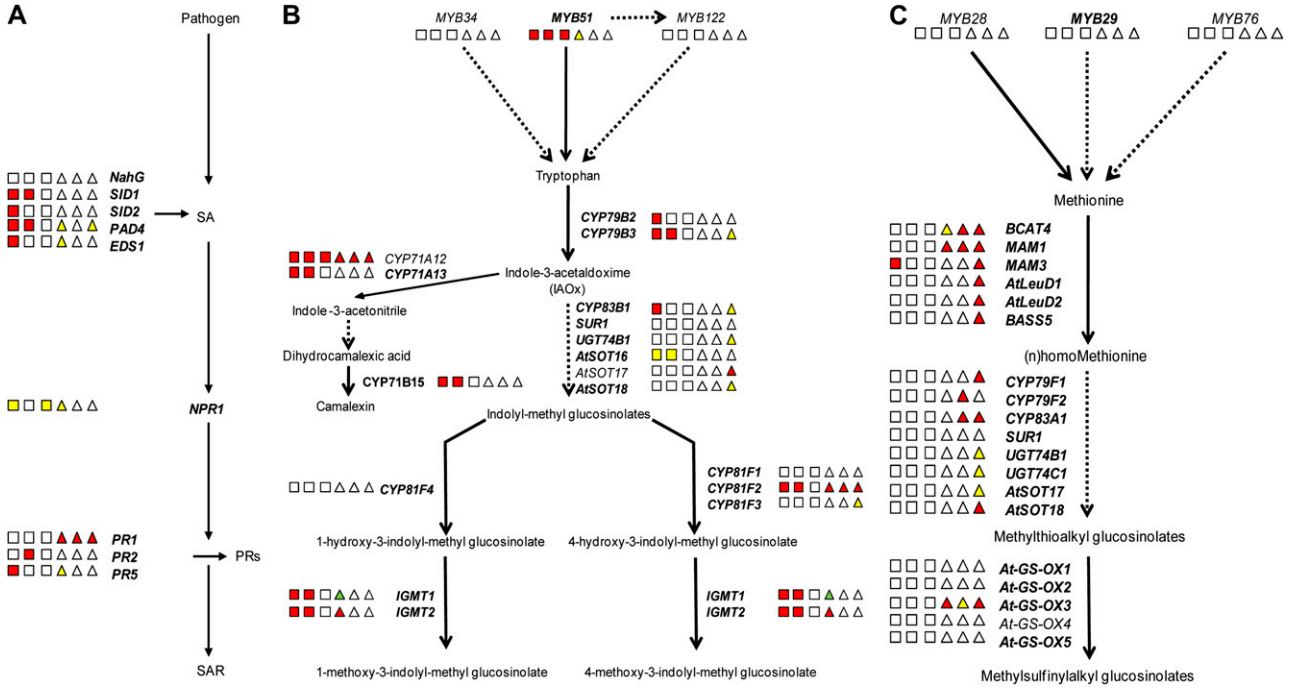

Figure 4. Selection of three major metabolic pathways induced in Arabidopsis plants by *Pf*.SS101. A, SA signaling pathway. B, Network involved in the biosynthesis of indole glucosinolates and camalexin. C, Network involved in the biosynthesis of aliphatic glucosinolates. Colored squares (leaves) and triangles (roots) represent statistically significant transcriptional changes induced in Arabidopsis by *Pf*.SS101 at 10, 14, or 18 DPI. Red = up-regulated; green = down-regulated; yellow = statistically significant transcriptional changes with fold changes less than 2.0; white = no statistically significant difference. Detailed information for each gene can be found in Table I.

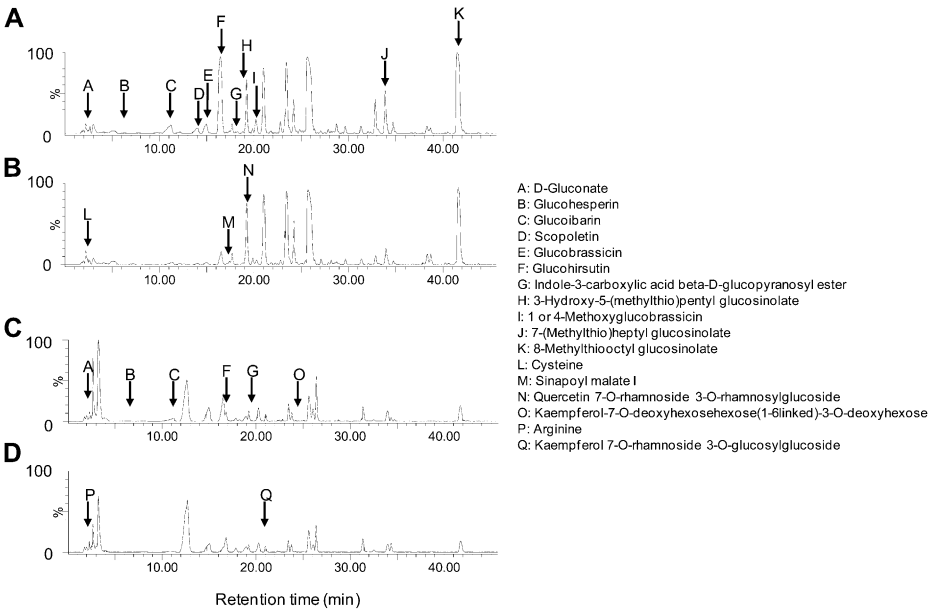

Figure 5. Untargeted LC-QTOF-MS-based metabolic profiling of Arabidopsis treated with *Pf*.SS101. A and B, LC-MS profiles of metabolites in the roots of Arabidopsis treated with *Pf*.SS101 (A) and mock-treated (control) Arabidopsis (B). C and D, LC-MS profiles of metabolites accumulated in the leaves of Arabidopsis treated with *Pf*.SS101 (C) and mock-treated (control) Arabidopsis (D). Prominent peaks that are significantly different (Student's *t* test, $P < 0.01$) between the control and *Pf*.SS101 treatment are labeled with letters. The entire data set and statistical analyses are presented in Supplemental Table S7.

To test the biological significance of the observed increases in the relative levels of glucosinolates and camalexin, *Pf*.SS101-treated plants and control plants of the R2R3 MYB transcription factor mutant *myb51*, mutants *cyp79B2cyp79B3*, *cyp81F2*, *pen2*, *cyp71A12*, *cyp71A13*, *cyp71B15* (*pad3*), and *myb28myb29*, were examined for their susceptibility/resistance toward *Pst*. The *cyp71B15* mock-treated mutant was significantly less susceptible ($P = 0.008$) to *Pst* than Col-0 (Fig. 6A), as was reported earlier (Glazebrook and Ausubel, 1994; Zhou et al., 1999). Additionally, a significant increase in susceptibility of the *myb51* mutant was observed in the mock-treated plants ($P = 0.013$) as well as in the *Pf*.SS101-treated plants ($P = 0.003$; Fig. 6A). The double mutant *myb28-myb29*, which is deficient in aliphatic glucosinolates (Beekwilder et al., 2008), was also more susceptible to *Pst* than Col-0 ($P = 0.018$). In all mutants except *cyp71B15*, *Pf*.SS101 could not induce resistance against *Pst* (Fig. 6A).

For the lepidopteran insect *S. exigua*, larval mortality was higher when feeding on Col-0 plants treated with *Pf*.SS101 as compared with nontreated plants (Fig. 6B). This difference was marginally significant ($P = 0.065$). A similar effect was recorded for larval mortality on the aliphatic glucosinolate mutant *myb28myb29* treated with *Pf*.SS101 ($P = 0.086$). In contrast, no difference in larval mortality of *S. exigua* was observed between the *Pf*.SS101-treated indolic glucosinolate mutant *cyp79B2cyp79B3* and the untreated control (Fig. 6B; $P = 0.934$). These data suggest that especially the indolic glucosinolates play a role in *Pf*.SS101-mediated resistance to this generalist insect herbivore.

DISCUSSION

In this study, we showed that colonization of Arabidopsis roots by the rhizobacterium *Pf*.SS101 leads to an enhanced level of resistance against various bacterial pathogens and the lepidopteran insect herbivore *S. exigua*. Our metabolomics and transcriptomics data showed that this specific rhizobacterial strain induces extensive metabolic and transcriptional changes in roots and leaves of Arabidopsis. Integration of the two omics data sets showed that, next to SA signaling, glucosinolates and camalexin play important roles in the resistance response of Arabidopsis induced by *Pf*.SS101.

Rhizobacteria-mediated ISR has frequently been shown to be dependent on JA/ET and independent of SA signaling (Pieterse et al., 1998; Ton et al., 2001; Yan et al., 2002; Van Wees et al., 2008; Van der Ent et al., 2009; Zamioudis and Pieterse, 2012). In contrast to these studies, we showed that the rhizobacterium *Pf*.SS101 induced resistance in Arabidopsis against *Pst* via the SA signaling pathway and not via the JA/ET pathways. Van der Ent and colleagues (2008) showed that colonization of Arabidopsis roots by nonpathogenic *Pf*.WCS417r triggered JA/ET-dependent ISR that is effective against a broad range of pathogens. Their microarray analysis revealed that the R2R3-MYB-like transcription factor gene *MYB72* was specifically activated in the roots upon colonization by *Pf*.WCS417r (Verhagen et al., 2004). They further showed that the T-DNA knockout mutants *myb72-1* and *myb72-2* were incapable of mounting ISR against the pathogens *Pst*, *H. parasitica*, *Alternaria brassicicola*, and *Botrytis cinerea*, indicating that MYB72 is essential to establish broad-spectrum ISR (Van der Ent et al., 2008). Here, we showed that *Pf*.SS101 did not affect the expression of *MYB72* and provided protection against *Pst* in the *myb72* knockout mutants to a level similar to that in wild-type Col-0 (Fig. 2C). Furthermore, *Pf*.SS101 was unable to induce resistance against *Pst* in mutants *npr1-1* and *npr1-3* (Fig. 2B). Mutant *npr1-1* is impaired in JA/ET/SA signaling, while mutant *npr1-3* is only

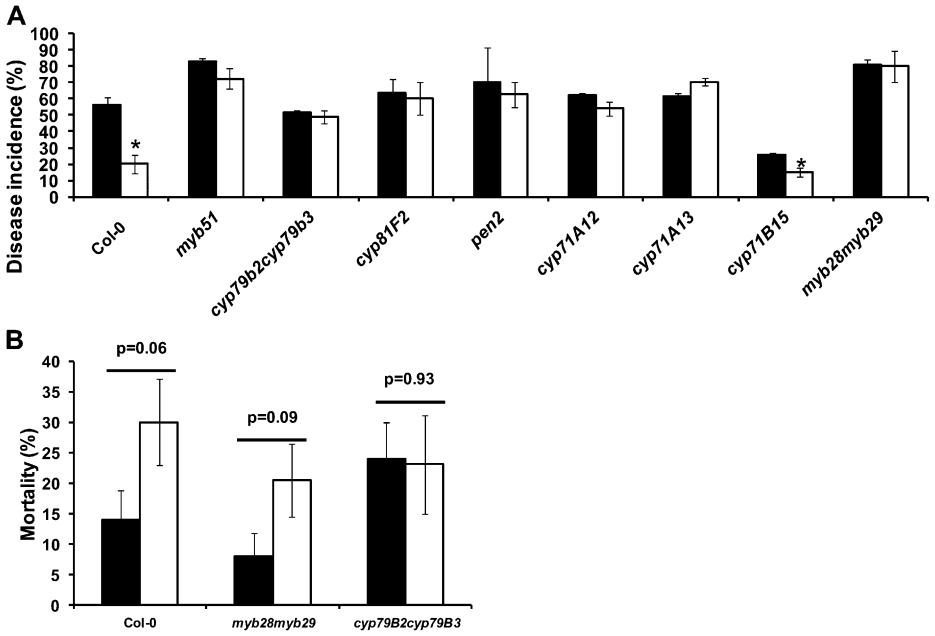

Figure 6. *Pf*.SS101 induces pathogen and insect resistance through the glucosinolate/camalexin biosynthesis pathways. A, Disease incidence of *Pst* on Arabidopsis wild type (Col-0) and glucosinolate/camalexin biosynthesis mutants grown in vitro. Symptoms were determined 5 d after inoculation. Values shown are means ± SE of four independent replicates of 10 plants each. Asterisks indicate statistically significant differences from the control treatment (one-way ANOVA, $P < 0.05$). B, Effects of *Pf*.SS101 on larval mortality of the lepidopteran insect *S. exigua* after 6 d of feeding. Values shown are back-transformed mortality means ± SE based on 13 to 25 plates per treatment (two-way GLM). Black bars represent mock-inoculated plants (control), and white bars represent *Pf*.SS101 root tip-inoculated plants.

implicated in SA signaling (Cao et al., 1994, 1997). Our analyses further showed significantly higher SA (free and bound) concentrations in roots of *Pf*.SS101-treated plants. In leaves, however, no differences in SA concentrations were detected between *Pf*.SS101-treated plants and nontreated plants (Fig. 2D). It is not yet known if and how the elevated SA concentrations in the roots of *Pf*.SS101-treated plants trigger the enhanced resistance to *Pst* in the leaves. Recently, Dempsey and Klessig (2012) suggested that a diverse group of compounds, including methylsalicylate, azelaic acid, the abietane diterpenoid dehydroabietinal, JA, and the amino acid derivative pipecolic acid, may act as mobile signals. Whether these signals play a role in *Pf*.SS101-induced resistance remains to be investigated. Altogether, we conclude that *Pf*.SS101 induces resistance dependent on SA signaling, thus differing from the signaling pathways induced by *Pf*.WCS417r and various other rhizobacterial strains.

Recently, Weston et al. (2012) used an integrated transcriptome and metabolome analysis to study the response of Arabidopsis to *P. fluorescens* strains GM30 and Pf-5. They showed a strain-specific response, with GM30-treated plants showing overrepresentation in genes for glucosinolate synthesis, abscisic acid, and gibberellin metabolism. Plants treated with Pf-5 showed enhanced expression of genes contributing to Trp degradation, auxin metabolism, and JA metabolism. When we compare the transcriptional changes induced by GM30 and Pf-5 with those induced by *Pf*.SS101, 442 genes were in common. In contrast, only 16 to 64 of the genes were shared between *Pf*.SS101 and other rhizobacteria (Supplemental Fig. S1; Supplemental Table S9; Cartieaux et al., 2003, 2008; Verhagen et al., 2004; Wang et al., 2005; Weston et al., 2012). From these data, we conclude that the signal transduction pathways and metabolic changes induced in Arabidopsis by *Pf*.SS101 do not resemble those induced by many other rhizobacteria studied to date.

Our results further showed that many of the defense-related genes that were up-regulated at 10 and 14 DPI in roots and leaves of *Pf*.SS101-treated Arabidopsis plants (Supplemental Tables S1 and S2) have also been described previously for pathogen-associated molecular pattern (PAMP)-triggered immunity (Thilmony et al., 2006). Expression analysis by Thilmony et al. (2006) of Arabidopsis plants treated with only avirulent *Pst* resulted in the identification of 735 PAMP-responsive genes, with 389 genes repressed and 346 genes induced. Of the 389 genes that were repressed upon PAMP perception, only 25 genes were repressed in the leaves of Arabidopsis plants treated with *Pf*.SS101. These results indicate that there is little overlap between the genes repressed by avirulent pathogens and by *Pf*.SS101. However, of the 346 genes that were induced upon PAMP perception by avirulent *Pst*, 195 genes were also induced in Arabidopsis leaves by *Pf*.SS101 (Supplemental Fig. S2A; Supplemental Table S10). When comparing the genes differentially expressed in Arabidopsis leaves by *Pf*.SS101 and virulent *Pst*, 134 genes are in common (Supplemental Fig. S2B; Supplemental Table S10). Of these 134 genes, 87 genes were induced and 47 genes were repressed by virulent *Pst* (Supplemental Fig. S2B; Supplemental Table S10). Thilmony et al. (2006) found that a total of 201 of the 346 PAMP genes induced by avirulent *Pst* were either repressed or not differentially regulated in response to virulent *Pst*. Based on these results, they postulated that these 201 genes may play a role in the basal defense response of Arabidopsis. More than one-half (108) of these 201 genes were significantly up-regulated in Arabidopsis leaves by *Pf*.SS101 at 10 or 14 DPI. These 108 genes may be considered as potential

candidates to further unravel the genetic basis of the defense response induced in Arabidopsis by *Pf*.SS101.

Many of the metabolic changes induced in Arabidopsis by *Pf*.SS101 are also similar to those induced in Arabidopsis leaves by avirulent *Pst-AvrRPM1*: more SA, camalexin, indole-3-carboxylic acid β-D-glucopyranosyl, and scopoletin accumulated in *Pst-AvrRPM1*-challenged leaves than in uninfected adjacent areas (Hagemeier et al., 2001; Simon et al., 2010). Additionally, *Pf*.SS101 induced the biosynthesis of indolic and aliphatic glucosinolates. Glucosinolates are well-studied plant metabolites with a key role in plant defense against microbial pathogens (Clay et al., 2009) and generalist herbivores from different feeding guilds (i.e. leaf chewers and phloem feeders; Mewis et al., 2006; Gols et al., 2008; Kim et al., 2008; Müller et al., 2010). Here, we show that glucosinolates are also an important component of the plant response to nonpathogenic, root-colonizing bacteria, which in turn affects resistance against pathogens and generalist herbivores. Furthermore, *Pf*.SS101 also induced the biosynthesis of various other metabolites not reported previously for Arabidopsis (Tohge et al., 2005; Malitsky et al., 2008; Albinsky et al., 2010;). Structural analysis of these unknown compounds and analysis of their activity spectrum have been initiated to resolve their functions in ISR.

The bacterial determinants involved in *Pf*.SS101-induced resistance in Arabidopsis remain unknown. Preliminary analyses indicated that massetolide A, the lipopeptide surfactant that contributes to induced systemic resistance in tomato against *P. infestans* (Tran et al., 2007), is not involved in the induced resistance response in Arabidopsis against *Pst*. This preliminary conclusion was based on assays with the *massA* mutant and purified massetolide A. Zamioudis and Pieterse (2012) indicated that beneficial microbes may be initially perceived by plants as potential invaders, whereas at later stages of the interaction the beneficial microbes "short-circuit plant defense responses to enable successful colonization of host roots." A possible scenario is that microbe-associated molecular patterns of *Pf*.SS101 and PAMPs of pathogens share specific structural features or are recognized in a similar manner, ultimately resulting in an enhanced defensive capacity of the plant. One potential candidate in this context is flagellin. Flg22 is a 22-amino acid polypeptide corresponding to a highly conserved epitope of the *Pseudomonas aeruginosa* flagellin protein (Felix et al., 1999) and is widely used as a proxy of flagellin-mediated signaling in Arabidopsis. In the genome of *Pf*.SS101 (Loper et al., 2012), we found a protein encoding a flagellin domain protein (PflSS101_3776; TRLSSGLKINSAKDDAAGMQIA) with an amino acid sequence similar to Flg22 in the N terminus (residues 30–51). This sequence has 95% identity to the *Pst* Flg22 sequence, with a difference of only one amino acid, and 80% identity to the *P. aeruginosa* Flg22 sequence, with a difference of four amino acids (Supplemental Fig. S3). Flagellin perception in Arabidopsis functions via the receptor-like kinase Flagellin-Sensing2 (FLS2; Zipfel et al., 2004). Hence, we investigated if FLS2 and also the EF-Tu Receptor (EFR) are involved in the resistance response induced by *Pf*.SS101. Stimulation of these receptors through their cognate ligands (i.e. bacterial flagellin or elongation factor Tu) leads to a defense response and increased resistance. The results of our bioassays showed that treatment of the mutants *fls2* and *efr* with *Pf*.SS101 led to a significant ($P < 0.05$) reduction in disease incidence to a similar level as observed for wild-type Arabidopsis Col-0 (Supplemental Fig. S4A). These results suggest that *Pf*.SS101 does not require FLS2 and EFR to induce resistance against *Pst* in Arabidopsis. To further support these results, we also studied callose deposition in *Pf*.SS101-treated plants. Flg22-elicited callose deposition in Arabidopsis cotyledons is dependent on the biosynthesis of indol-3-ylmethylglucosinolate, which in turn is dependent on the transcription factor MYB51 (Clay et al., 2009). In our bioassays, we observed that root tip inoculation with *Pf*.SS101 did not cause callose deposition in Arabidopsis leaves (Supplemental Fig. S4B). Furthermore, callose deposition was also not induced in leaves when *Pf*.SS101-treated Arabidopsis plants were challenged with *Pst* (Supplemental Fig. S4B) or induced in roots of Arabidopsis treated with *Pf*.SS101 (data not shown).

In silico analysis of the genome sequence of *Pf*.SS101 showed the presence of a type III secretion system (TTSS; Loper et al., 2012). TTSS is known to function in the delivery of effector molecules into the plant and plays an important role in the pathogenicity/virulence of bacterial pathogens. For *P. fluorescens* strain SBW25, the *tts* cluster harbors several open reading frames that share a significant degree of similarity with the *hrc/hrp* genes of pathogenic bacteria (Preston et al., 2001). Also, the TTSS of *Pf*.SS101 resembles the *hrc/hrp* genes of *P. syringae* and carries a rhizosphere-expressed secretion protein and an rsp-conserved (*rsp/rsc*) gene cluster (Loper et al., 2012). Furthermore, the *Pf*.SS101 genome harbors 12 additional genes preceded by putative Hrp(Rsp)L-dependent promoters and encoding proteins with compositions typical of type III effectors. The potential role of these putative novel effectors in induced systemic resistance by *Pf*.SS101 is the subject of future studies.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Pseudomonas fluorescens SS101 (*Pf*.SS101) was cultured in liquid King's medium B (KB) at 25°C for 24 h. Bacterial cells were collected by centrifugation, washed three times with 10 mM $MgSO_4$, and resuspended in 10 mM $MgSO_4$ to a final density of 10^9 CFU mL^{-1} (optical density at 600 nm = 1.0). *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*) was cultured in KB broth supplemented with rifampicin (50 μg mL^{-1}) at 25°C for 24 h. *Pseudomonas syringae* pv *maculicola*, *Pseudomonas alisalensis*, and *Pseudomonas viridiflava* were cultured in KB broth at 25°C for 24 h. Bacterial cells were collected by centrifugation, washed three times in 10 mM $MgSO_4$, and resuspended in 10 mM $MgSO_4$ to a final density of 10^9 CFU mL^{-1} for inoculating plants.

Plant Material, Pathogen Challenge, and Insect Feeding

The T-DNA insertion lines of Arabidopsis (*Arabidopsis thaliana*) were obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003) and GABI-Kat (Rosso et al., 2003). PCR was performed by using the T-DNA left-border primer (LBa1 for SALK lines, 5′-TGGTTCACGTAGTGGGC-CATCG-3′, and GABI_08409 for Gabi_Kat lines, 5′-ATATTGACCATCA-TACTCATTGC-3′) and genomic sequences that correspond to the flanking DNA to identify the mutant allele or both of the genomic primers to identify the wild-type allele (Supplemental Table S11).

Seeds of Arabidopsis Col-0 and the various mutants were surface sterilized for 3 h by placing seeds in opened Eppendorf tubes in a desiccator jar. Two 100-mL beakers each containing 50 mL of commercial bleach were placed inside, and 1.5 mL of concentrated HCl was added to each beaker. The desiccator jar was closed, and the seeds were sterilized by chlorine gas. After 4 h, seeds were sown in petri dishes on water-saturated filter paper followed by a 3-d treatment at 4°C. Thereafter, 10 to 12 seeds were sown on plates containing 50 mL of one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962). One-week-old Arabidopsis seedlings were root tip inoculated with 2 μL of *Pf*.SS101 cell suspensions (10^9 CFU mL^{-1}); in the control treatment, seedlings were inoculated with 2 μL of 10 mM $MgSO_4$. After an additional 3 d of plant growth, the 10-d-old seedlings were transferred to 60-mL polyvinyl chloride pots containing a sand-potting soil mixture that was autoclaved twice for 20 min with a 24-h interval. Once a week, plants were supplied with modified one-half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938). For the induced resistance assays conducted in soil, 18-d-old plants were challenged by spray inoculating the leaves with a cell suspension of *Pst* at 2.5×10^7 CFU mL^{-1} in 10 mM $MgSO_4$ supplemented with 0.01% (v/v) Silwet L77. One day before challenge inoculation, the plants were placed at 100% relative humidity. Six days after challenge inoculation, disease incidence was assessed by determining the percentage of diseased leaves per plant. Leaves were scored as diseased when they exhibited necrotic or water-soaked lesions surrounded by chlorotic tissue. From the number of diseased and nondiseased leaves, the disease incidence was calculated for each plant (20–30 plants per treatment). The experiment was performed at least twice. Colonization levels of the rifampicin-resistant *Pst* and *Pf*.SS101 bacteria were determined at the end of bioassays with the method described by Pieterse et al. (1996). Statistically significant differences were determined by ANOVA ($P < 0.05$).

In the in vitro assays on one-half-strength Murashige and Skoog medium, *Pf*.SS101 was applied to the seeds or to the root tips. For both treatments, seeds were sterilized and sown on plates as described above. Germination and plant cultivation were performed in a climate chamber (21°C/21°C day/night temperatures; 250 μmol m^{-2} s^{-1} at plant level during 16 h d^{-1}; 70% relative humidity). For the *Pf*.SS101 seed treatment, a cell suspension (10^9 CFU mL^{-1}) of *Pf*.SS101 was added to the sterilized seeds in a petri dish and incubated for 30 min at room temperature. For the control, seeds were incubated for 30 min with sterile 10 mM $MgSO_4$. For the root tip treatment, 2 μL of *Pf*.SS101 (10^9 CFU mL^{-1}) was spot inoculated on root tips of 1-week-old seedlings. Control plants were inoculated with 2 μL of 10 mM $MgSO_4$. The challenge with *Pst* and other bacterial pathogens was performed by inoculation of a 2-μL cell suspension (10^9 CFU mL^{-1}) in the center of the leaf rosette of 14-d-old plants. Five to 7 d after challenge inoculation, disease incidence was assessed by determining the percentage of diseased leaves per plant as described above.

Spodoptera exigua was reared on an artificial diet in a climate chamber (27°C ± 2°C, 70%–80% relative humidity, 16-h-light/8-h-dark cycle) according to Smits et al. (1986). For the insect assays, one to two neonate larvae of *S. exigua* were transferred to one plate containing 10 18-d-old plants. After 6 d of incubation in the growth chamber, insect mortality was recorded. The assay with wild-type Col-0 (Fig. 1C) was repeated twice, and in total, 45 to 48 plates were used for *Pf*.SS101-treated or untreated plants. A total of 13 to 25 plates per treatment were evaluated in the assay with several Arabidopsis lines (Fig. 6B). The effect of *Pf*.SS101 treatment on insect mortality was analyzed using a generalized linear model (GLM) with logit link function and binomial distribution, and the dispersion parameter was estimated to correct for overdispersion (GenStat 13).

RNA Extraction and Microarray Analysis

Total RNA was extracted from roots and shoots of untreated and *Pf*.SS101-treated plants after 10, 14, and 18 d of growth. Three biological replicates of 30 plants per replicate were used for each time point. RNA was isolated from the frozen tissues with Trizol reagent (Invitrogen). The RNA samples were further purified using the NucleoSpin RNA II kit (Macherey-Nagel). For the Affymetrix Arabidopsis genome GeneChip array analysis (ServiceXS), amplification and labeling of the RNA samples as well as hybridization, staining, and scanning were performed according to the manufacturer's specifications.

Microarray Data Analyses

Bioconductor packages (Gentleman et al., 2004) were used to analyze the scanned Affymetrix arrays. The Bioconductor packages were integrated in the automated online MADMAX pipeline. The arrays were normalized using quantile normalization, and expression estimates were compiled using robust multiarray average applying the empirical Bayes approach (Wu et al., 2004). They were considered of sufficiently high quality if (1) they showed less than 10% of specks in fitPLM model images, (2) they were not deviating in RNA degradation and density plots, (3) they were not significantly deviating in NUSE and RLE plots, and (4) they were within each other's range in box plots. Differentially expressed probe sets were identified using linear models, applying moderated *t* statistics that implement empirical Bayes regularization of SE values (Smyth, 2004). Venn diagrams and basic comparisons were made in Microsoft Excel.

Quantitative PCR Analysis

To confirm and extend the results obtained in the genome-wide microarray analyses, quantitative PCR analysis was performed on RNA isolated from roots and leaves of Arabidopsis plants grown in vitro for 10, 14, and 18 d as described above. Four biological replicates containing 10 to 12 plants each were used for each time point. The plant tissues were frozen in liquid N_2 and stored at –80°C. RNA was isolated from the frozen tissues with Trizol reagent (Invitrogen) followed by DNase I (GE Healthcare) treatment. One microgram of RNA was used for copy DNA synthesis with SuperScript III (Invitrogen) according to the manufacturer's protocol. Quantitative PCR was conducted with the 7300SDS system from Applied Biosystems and the SensiMix SYBR kit (Bioline) with a final concentration of 3.0 mM $MgCl_2$. The primers used for the quantitative PCR are listed in Supplemental Table S12. The primer concentrations were optimized (400 nM final concentration for all), and a dissociation curve was obtained to check the specificity of the primers. To correct for small differences in template concentration, *UBQ10* was used as a reference. The cycle in which the SYBR Green fluorescence crosses a manually set threshold cycle (C_T) was used to determine transcript levels. For each gene, the threshold was fixed based on the exponential segment of the PCR curve. The C_T value for the gene of interest was corrected for the reference gene as follows: $\Delta C_T = C_{T(gene)} - C_{T(UBQ10)}$. The relative quantification (RQ) values were calculated by the formula $RQ = 2^{-[\Delta CT(\text{treatment day 10, 14 or 18}) - \Delta CT(\text{control day 10, 14, 18})]}$. If there is no difference in transcript level between the *Pf*.SS101 treatment and the control, then RQ = 1 (2°) and log RQ = 0. Statistically significant differences were determined for log-transformed RQ values by ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference posthoc multiple comparison.

Measurement of SA Levels (Free and 2-*O*-β-D-Glucoside)

SA extraction from 150 mg fresh weight of roots and leaves was performed according to Verberne et al. (2002) using 2-hydroxybenzoic-acid-d6 as an internal standard. A LC-PDA-Orbitrap FTMS system (Thermo) at 60,000 resolution in negative ionization mode (Van der Hooft et al., 2012) was used to specifically detect the accurate masses of SA (mass-to-charge ratio $[M\text{-}H]^-$ = 137.0244) and the internal standard (detected as mass-to-charge ratio $[M\text{-}H]^-$ = 141.0495).

Untargeted Metabolomics Analysis

After 18 d of plant growth, leaves and roots from three biological samples, each containing 100 untreated or 100 *Pf*.SS101-treated plants, were snap frozen in liquid nitrogen and ground to a fine powder under continuous cooling. Metabolite profiling of roots and leaves was performed using accurate mass LC-quadrupole time-of-flight (QTOF)-MS (De Vos et al., 2007). In short, 100 mg fresh weight of plant material was extracted in 1 mL of 75% (v/v) aqueous methanol containing 0.1% (v/v) formic acid. The extracts were sonicated for 15 min, centrifuged (20,000*g*) for 10 min, transferred to 96-well protein filtration plates (Captiva 0.45 m; Ansys Technologies), vacuum

filtrated, and collected in 700-μL glass inserts on 96-well autosampler plates (Waters) using a Genesis Workstation (Tecan Systems). Extracts (3 μL) were injected in an Alliance HPLC system, and metabolites were detected with a PDA detector coupled to a high-resolution QTOF Ultima mass spectrometer (Waters). Electrospray ionization in negative mode was used to ionize compounds separated by the reverse-phase C18 column (150 $\times$ 2.1 mm, 3 μm; Phenomenex). Raw data were processed in an untargeted manner by extracting and aligning all mass signals with a signal-to-noise ratio of at least 3 across all samples using the dedicated Metalign software (www.metalign.nl). This resulted in a data matrix of intensities of 2,491 mass signals $\times$ 12 samples. Low-mass signals not exceeding an intensity of greater than 10 times the noise in any sample were filtered out, leaving 980 mass features. All analyses were performed with three biological replicates, and the average variation between replicate analyses in intensities of the 980 mass signals was 6.17%, with more than 95% of the signals varying less than 20%. MSClust software (Tikunov et al., 2012) was subsequently used to group individual mass signals into 129 reconstructed metabolites. Student's *t* tests were applied to identify mass signals that were significantly ($P < 0.01$) changed between *Pf*.SS101-treated and control samples.

Annotation of differential metabolites was based on selecting the pseudomolecular ions from the masses in the MSClust-reconstructed metabolites, first, by matching their accurate masses plus retention times to previously reported Arabidopsis metabolites analyzed on the same LC-MS system and with the same chromatographic conditions (Beekwilder et al., 2008; Stracke et al., 2009; Van der Hooft et al., 2012). Second, if compounds were not yet present in this experimentally obtained database, detected masses were matched with natural compound libraries, including Metabolomics Japan (http://metabolomics.jp) and the Dictionary of Natural Products (http://dnp.chemnetbase.com), using a maximum deviation of observed mass from calculated mass of 5 ppm and with first focus on metabolites that have been earlier described in Arabidopsis or other Brassicaceae species. Candidate structures were checked for corresponding in-source fragments and UV/visible absorbance characteristics, if present, and by comparing with commercially available authentic standards.

Callose Deposition

To visualize callose deposition, Arabidopsis was grown for 18 d with or without *Pf*.SS101. After 18 d, leaves were inoculated with 2 μL of *P. syringae* hrcC, *P. syringae* avrRPM1, or *Pst* cell suspension (10^9 CFU mL^{-1}); in the control treatment, leaves were inoculated with 2 μL of 10 mM $MgSO_4$. Leaves were harvested 4 d later, cleared overnight in 70% ethanol, and stained with 1% (w/v) aniline blue in 150 mM K_2HPO_4 (pH 9.5) for 1 h. Fluorescent callose deposits were viewed using a Nikon 90i epifluorescence microscope with an appropriate filler. Images of randomly selected fields were captured using a Nikon DS-5Mc digital camera.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Venn diagram of differentially regulated Arabidopsis genes upon treatment with *Pf*.SS101 or other nonpathogenic rhizobacterial strains.

Supplemental Figure S2. Venn diagram of differentially regulated Arabidopsis genes upon treatment with *Pf*.SS101 or avirulent and virulent *Pst*.

Supplemental Figure S3. Comparison of Flg22 and Flg22-derived amino acid sequences from *Pf*.SS101, *Pst*, and *P. aeruginosa* PA7.

Supplemental Figure S4. Effect of *Pf*.SS101 on the resistance of Arabidopsis wild type and the FLS2 and EFR mutants against *Pst* and on callose deposition.

Supplemental Table S1. Differentially expressed genes in Arabidopsis roots treated with *Pf*.SS101.

Supplemental Table S2. Differentially expressed genes in Arabidopsis leaves treated with *Pf*.SS101.

Supplemental Table S3. ORA of genes that are up-regulated in the roots of *Pf*.SS101-treated Arabidopsis at 10, 14, or 18 DPI .

Supplemental Table S4. ORA of genes that are down-regulated in the roots of *Pf*.SS101-treated Arabidopsis at 10, 14, or 18 DPI.

Supplemental Table S5. ORA of genes that are up-regulated in the leaves of *Pf*.SS101-treated Arabidopsis at 10, 14, or 18 DPI.

Supplemental Table S6. ORA of genes that are down-regulated in the leaves of *Pf*.SS101-treated Arabidopsis at 10, 14, or 18 DPI.

Supplemental Table S7. Metabolites significantly changed in Arabidopsis plants treated with *Pf*.SS101.

Supplemental Table S8. Quantitative PCR analysis of genes involved in glucosinolate and camalexin biosynthesis.

Supplemental Table S9. List of genes differentially regulated in Arabidopsis in response to *Pf*.SS101 and other nonpathogenic bacteria.

Supplemental Table S10. List of genes differentially regulated in Arabidopsis in response to *Pf*.SS101 and *P. syringae*.

Supplemental Table S11. Primers used for the identification of homozygous T-DNA insertion lines.

Supplemental Table S12. Primers used for quantitative PCR.

ACKNOWLEDGMENTS

We thank Bart Thomma for providing the seeds of the *ein2*, *jar1*, *nahG*, *npr1-1*, *npr1-3*, *myb72*, *myc2*, *pen2*, and *coi1-16* mutants and Jules Beekwilder for providing the seeds of the *myb28myb29* mutant.

Received September 13, 2012; accepted October 16, 2012; published October 16, 2012.

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