Effect of Wheat Roots Infected with the Pathogenic Fungus *Gaeumannomyces graminis* var. *tritici* on Gene Expression of the Biocontrol Bacterium *Pseudomonas fluorescens* Pf29Arp

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Traits contributing to the competence of biocontrol bacteria to colonize plant roots are often induced in the rhizosphere in response to plant components. These interactions have been studied using the two partners in gnotobiotic systems. However, in nature, beneficial or pathogenic fungi often colonize roots. Influence of these plant–fungus interactions on bacterial behavior remains to be investigated. Here, we have examined the influence of colonization of wheat roots by the take-all fungus *Gaeumannomyces graminis* var. *tritici* on gene expression of the biocontrol bacterium *Pseudomonas fluorescens* Pf29Arp. Bacteria were inoculated onto healthy, early *G. graminis* var. *tritici*-colonized and necrotic roots and transcriptomes were compared by shotgun DNA microarray. Pf29Arp decreased disease severity when inoculated before the onset of necrosis. Necrotic roots exerted a broader effect on gene expression compared with early *G. graminis* var. *tritici*-colonized and healthy roots. A gene encoding a putative type VI secretion system effector was only induced in necrotic conditions. A common pool of Pf29Arp genes differentially expressed on *G. graminis* var. *tritici*-colonized roots was related to carbon metabolism and oxidative stress, with a highest fold-change with necrosis. Overall, the data showed that the association of the pathogenic fungus with the roots strongly altered Pf29Arp adaptation with differences between early and late *G. graminis* var. *tritici* infection steps.

The rhizosphere, usually defined as the area influenced by the plant root system, provides a nutrient-rich environment for microorganisms. However, in the soil environment, roots are commonly colonized with filamentous fungi that may be beneficial or pathogenic in nature (de Boer et al. 2005). The influence of roots and fungi creates an environment that differs from the rhizosphere of an uninfected plant. For example, the mycorrhizosphere, which is the area influenced by both roots and mycorrhizal fungus, is colonized by a modified bacterial

community compared with the rhizosphere (Andrade et al. 1997; Frey-Klett et al. 2005). A similar effect occurs in the pathorhizosphere, as the result of roots infected with a pathogenic fungus (Chapon et al. 2002). Indeed, the healthiest plant may not provide the greatest benefits in terms of fitness for many rhizobacteria (Denison et al. 2003). Lesions caused by soil fungal pathogens can increase nutrient availability in the rhizosphere due to leakage from damaged root tissues (Curl and Truelove 1986) leading to the selection of specific rhizobacteria community (Sanguin et al. 2008).

Certain rhizobacteria exert positive effects on plant fitness by reducing fungal soilborne diseases and improving plant growth. Such bacteria belong to the plant-growth-promoting rhizobacteria (PGPR) group, which includes many genera, such as *Pseudomonas* (Kloepper et al. 1980). The main biocontrol mechanisms are active exclusion of pathogenic fungi from the rhizosphere (Haas and Defago 2005) and induction of plant disease resistance (van Loon et al. 1998). The efficiency of biocontrol bacteria depends not only on their direct and indirect fungistatic or fungicidal activity but also on their root colonization capacity, which contributes to rhizosphere competence (Lugtenberg et al. 2001). Various bacterial functions, such as chemotaxis (de Weert et al. 2002), motility (Simons et al. 1996; Ramos-Gonzalez et al. 2005), cell attachment (Buell and Anderson 1992; Rodriguez-Navarro et al. 2007), growth (Simons et al. 1997; Rainey 1999), microcolony formation (Chin-A-Woeng et al. 1997; Bloemberg et al. 2000), and stress resistance (Sarniguet et al. 1995; Matilla et al. 2007) were proven to be involved in rhizosphere competence and in improvement of root colonization. Root exudates such as organic acids, sugars, and amino acids influence the ability of microbial strains to colonize the roots (Mark et al. 2005; Kamilova et al. 2006a). However, pathogenic fungi modify the amounts and composition of root exudates components (Kamilova et al. 2006b). Furthermore, fungi are also expected to play a major role in bacterial growth and movement in soil (Tarkka et al. 2009); some hyphal exudates induce bacterial chemotaxis and growth (de Weert et al. 2004) and hyphae can act as a surface to promote movement of soil bacteria (Kohlmeier et al. 2005). For example, the biocontrol strain *Pseudomonas fluorescens* WCS365 preferentially colonizes hyphae of the pathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* in the tomato rhizosphere (Bolwerk et al. 2003). However, none of

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these studies have explored the impact of pathogenic fungi or fungal-infected roots on the molecular determinants involved in root colonization and biocontrol efficiency of PGPR.

The decrease of take-all disease severity after several years of wheat monoculture is related to the selection of biocontrol rhizobacteria, including pseudomonads (Cook and Rovira 1976; Sanguin et al. 2008). This phenomenon, called take-all decline, needs a severe outbreak caused by the pathogenic fungus *Gaeumannomyces graminis* var. *tritici*. An effect of roots colonized by *G. graminis* var. *tritici* on the composition and the amount of biocontrol pseudomonad populations is observed (Chapon et al. 2002). The presence of *G. graminis* var. *tritici* and the necrotic root lesions caused by *G. graminis* var. *tritici* drive the development of populations of certain types of pseudomonad (Barnett et al. 1999). For instance, the strain Pf29Arp, which belongs to a specific repetitive extragenic palindromic polymerase chain reaction (REP-PCR) cluster and is an efficient wheat root colonizer in the absence of *G. graminis* var. *tritici*, becomes dominant toward indigenous pseudomonads in the presence of *G. graminis* var. *tritici* and decreases take-all severity when inoculated in soil (Sarniguet et al. 1997; Chapon et al. 2002). Three mechanisms of biocontrol were suggested: environmental acidification through gluconic acid production, induced systemic plant resistance, and change in microbial community composition (Sarniguet et al. 2006). In

previous work, the influence of *G. graminis* var. *tritici* alone on Pf29Arp gene expression was assessed during a commensal in vitro interaction (Barret et al. 2009). However, the influence of roots infected with pathogenic fungus on bacterial gene expression remains unknown.

The aim of the present work was to assess the effect of wheat roots infected with *G. graminis* var. *tritici* on gene regulations of the biocontrol strain *P. fluorescens* Pf29Arp. For this purpose, Pf29Arp was inoculated in three different environments: healthy roots, early colonized roots, with *G. graminis* var. *tritici* and necrotic roots. Bacterial transcriptome was compared with the use of a Pf29Arp genomic shotgun array (Barret et al. 2009).

RESULTS

Colonization patterns of wheat roots by *G. graminis* var. *tritici*.

To describe the infection process of *G. graminis* var. *tritici* in the in vitro bioassay (Fig. 1), we performed a time-course measure of size of necrotic lesions (Fig. 2) and a simultaneous microscopic observation of the fungal mycelium after staining with Chlorazol Black E (Fig. 3). Early root colonization and infection events were observed on 7-day-old wheat seedlings inoculated with the strain *G. graminis* var. *tritici* IV-26/00. Two days after inoculation with *G. graminis* var. *tritici* (day 9), fungal plugs were removed and hyphae were observed on the root surface (Fig. 3G). At day 9, no necrosis symptoms were observed but the pathogen had already penetrated the host tissue through the root epidermis because hyphae were present inside some root cortex cells (Fig. 3B). At day 10, small 1-mm necroses were visible (Fig. 2). At day 11, necrosis size reached 3 mm (Fig. 2) and hyphae were present in all root cortex cells (Fig. 3F). At day 12, necrosis extended to 6 mm and hyphae of *G. graminis* var. *tritici* were more abundant in the cortex cells and caused host tissue alteration. The hyphae also passed the endodermis and entered the stele tissue of the roots. At day 14, necrosis reached 10 mm, the pathogen completely colonized the stele tissue (Fig. 3D), and *G. graminis* var. *tritici* hyphae were also observed in xylem vessels (Fig. 3E). At this stage, the massive colonization of the roots caused marked cells degradation.

Effects of Pf29Arp on *G. graminis* var. *tritici* pathogenesis.

The influence of Pf29Arp on *G. graminis* var. *tritici* pathogenesis in the in vitro bioassay was assessed by severity (corresponding to the necrosis length of diseased roots). Two Pf29Arp inoculation treatments, one at day 9 (early Pf29Arp treatment) before onset of necrosis (E) and a second at day 12 (late Pf29Arp treatment) after onset of necrosis (N) were compared with the *G. graminis* var. *tritici* inoculation treatment without bacteria at the same corresponding stages (Fig. 4).

At day 11, disease severity of necrotic roots was not significantly different in *G. graminis* var. *tritici* control (mean necrosis length of 2.3 ± 0.4 mm) and early Pf29Arp treatments (0.8 ± 0.3). At day 12 and 14, severity was not significantly different between *G. graminis* var. *tritici* control (4.6 ± 0.6 ; 10.3 ± 0.5) and late (4.6 ± 0.6 ; 9.0 ± 0.7) Pf29Arp treatments. However, at these same days, severity was significantly lower for early Pf29Arp treatment (1.8 ± 0.6 ; 3.1 ± 0.8) compared with the two other treatments ($P = 0.05$).

Disease incidence (expressed as percentage of diseased roots) was also assessed (data not shown). At day 11 and 12, 69.6 and 78.3%, of *G. graminis* var. *tritici* control roots had necrosis, respectively, while incidence dropped significantly to 30.4% for early Pf29Arp treatment according to confidence interval (IC) ($P = 0.05$; $\Delta = 39.1$, IC_{11d} [1.7, 77.6]; $\Delta = 47.8$,

Fig. 1. Scheme of the bioassay used to arrange tripartite interaction between wheat roots, the pathogenic fungus *Gaeumannomyces graminis* var. *tritici* strain IV-26/00 and the bacterial strain *Pseudomonas fluorescens* Pf29Arp. Roots were placed on water agar of a 9-cm petri dish containing water agar (30 g liter⁻¹). A hole was previously made at the border and also in the lid of the dish to let the root grow gnotobiotically inside and to allow the leaf to grow outside. Fungal plugs (■) of strain *G. graminis* var. *tritici* IV-26/00 were placed on each principal roots at 3 cm from the seed and removed after 2 days. Pf29Arp (10^6 cells) were deposited on each root at 3 cm from the seed. Each dish was closed with Parafilm M and surrounded with an aluminum sheet to make an opaque environment. Then dishes were placed inside a larger 15-cm sterile dish to retain a sterile environment. The combined plates were then incubated at 15°C with an alternating cycle of 14 h of light and 10 h of darkness.

Fig. 2. Time-course of *Gaeumannomyces graminis* var. *tritici* infection of wheat roots. Roots were observed under a binocular microscope at day 7, 8, 9, 10, 11, 12, and 14. Plugs cut from the growing margin of a *G. graminis* var. *tritici* IV-26/00 culture were inoculated at either day 7 or day 10 on roots and removed 2 days later. At day 12, 10^6 cells of *Pseudomonas fluorescens* Pf29Arp were inoculated on healthy, early *G. graminis* var. *tritici*-colonized or necrotic roots, respectively. At day 14, roots were excised and bacteria were harvested. Length of necrosis was measured with the Archimed software (Microvision instruments, Evry, France).

IC_{12d} [11.4, 84.3]). At day 14, 100% of *G. graminis* var. *tritici* control roots had necrosis while incidence decreased significantly to 78.3% for early Pf29Arp treatment ($\Delta = 21.7$, IC_{14d} [4.5, 39.0]). However, microscopic observation of roots colonized with both *G. graminis* var. *tritici* and Pf29Arp at day 12 and 14 revealed few hyphae inside the cortical cells for non-necrotic roots (data not shown). On the contrary, late Pf29Arp treatment had no significant effect on disease incidence compared with *G. graminis* var. *tritici* control roots.

Pf29Arp colonization patterns.

P. fluorescens Pf29Arp growth was monitored by dilution plating (Fig. 5). Although Pf29Arp inoculation was made from the same bacterial suspension, the number of CFU per centimeter of roots at time-point 0 (20 min after Pf29Arp inoculation) differed significantly ($P = 0.05$) for necrotic roots (N) when compared with healthy (H) and early *G. graminis* var. *tritici*-colonized roots (E). During the first 24 h, the population density increased similarly for N and H treatments with a respective log₁₀ bacterial multiplication rate of 1.0 (± 0.1) and 1.2 (± 0.2) and was significantly (four times) less with a rate of 0.3 (± 0.1) in treatment E. Population density of N and E treatments still increased between 24 and 48 h with a bacterial multiplication rate of 0.7 (± 0.1) and 0.6 (± 0.1), respectively, whereas population density of H treatment remained constant.

At 24 h for H and at 48 h for E and N, population amount reached approximately 5×10^6 cells for the three treatments. After 48 h, cells density remained constant for the three treatments until 96 h, with only a slight decrease observed for treatment E between 72 and 96 h (-0.31 ± 0.1).

Microarray hybridization.

Pairs of Pf29Arp RNA samples were extracted from healthy roots (H), roots early colonized with *G. graminis* var. *tritici* without any necrosis (E), and necrotic roots (N) 2 days after bacterial inoculation. After RNA amplification, the corresponding cDNA were then hybridized to different microarray slides following a loop design. Three independent biological replicates, each containing two technical replicates (dye-swap), were performed for each experiment.

Range of RNA amplification bias effects were examined in determining the number of genes over- or under-repressed in amplified compared with unamplified expression profiles (Supplementary Fig. S1). Using an arbitrary log₂ 0.3-fold cut-off, we detected 874 genomic regions over- or under-represented (8.2% of the spots after normalization). However, these differences will not significantly alter genomics regions with high expression ratios ($-1 \leq \log_2 \leq 1$). Therefore, this amplification method (Moreno-Paz and Parro 2006) was validated for further transcriptional analyses.

Fig. 3. Colonization stages of wheat roots by *Gaeumannomyces graminis* var. *tritici* IV-26/00. Roots of 7 day-old seedlings were inoculated with 8-mm³ (2 by 2 by 2 mm) agar plugs containing *G. graminis* var. *tritici* IV-26/00 mycelium. Colonization of the root surface and tissues was observed with light microscopy at **B** and **G**, day 9; **F** and **H**, day 11; **C**, day 12; and **A**, **D**, **E**, and **I**, day 14. **A**, Cross-section of healthy wheat root. **B**, Cross-section of a wheat root one-sided attack by *G. graminis* var. *tritici*; the left part of the cortex was filled with mycelium whereas the right part is unaffected. **F**, Penetration of one hypha (arrow) was visible at junction between two cortical cells. **C**, Hyphae proliferated within root cortical cells, filling most of the intracellular space and progressed through the endodermis and reached the stele. **D** and **E**, Hyphae colonized xylem tubes and the root cortical tissues were destroyed. Melanized runner hyphae (arrow) grew along the root surface **G**, before and **H**, after onset of necrosis. **I**, Infection hyphae are constricted (arrow) when passing through the cell walls. Bars = 100 μ m (**A**, **B**, **C**, **D**, and **H**); 50 μ m (**E**); 25 μ m (**F** and **G**); and 10 μ m (**I**).

According to the F test ($P < 0.01$), in total, 3,077 differentially expressed genes were detected in pairwise comparisons of the following growth conditions: E versus H, N versus H, and N versus E (Fig. 6). Most differentially expressed genomic regions ($n = 3,004$) were observed in N versus H, whereas 822 and 698 genomic regions were differentially regulated in E versus H and N versus E, respectively. In total, 1,719, 31, and 38 differentially expressed genomic regions were exclusively found in N versus H, E versus H, and N versus E, respectively. Because of the loop design, we expected that expressed genomic regions would be distributed in two pairwise comparisons. How-

Fig. 4. Effect of *Pseudomonas fluorescens* Pf29Arp on take-all severity. Wheat seedlings grown in water agar were inoculated with *Gaeumannomyces graminis* var. *tritici* IV-26/00. Seedlings were treated with *P. fluorescens* Pf29Arp at day 9 (before onset of necrosis) or at day 12 (after onset of necrosis). The length of necrosis was calculated from 23 roots at days 11 (white bars), 12 (gray bars), and 14 (black bars). Differences between treatments were analyzed by analysis of variance and were significant at $P = 0.05$.

ever, the majority of genes exclusively found in one comparison had small fold-change (means: -0.31 and 0.34) explaining while they were not found in other comparison.

On the 789 (627 ± 162) genomic regions shared between N versus H and E versus H which were specific to bacterial transcriptome response to the fungal presence on roots, 774 shared the same expression pattern (data not shown), with a slight increase in expression ratio for N versus H compared with E versus H (either up- or downregulated), which was significant for 160 genes present in the intersection of the three comparisons. The 658 (162 ± 496) differentially expressed genomic regions shared only between N versus E and N versus H were specific to bacterial transcriptome response to lesions on roots with 650 genes similarly regulated. Among these 650 genes, a significant increase in expression ratio of 159 genes was found for N versus H compared with N versus E. Finally, 164 (162 ± 2) genomic regions were specifically shared between E versus

Fig. 5. *Pseudomonas fluorescens* Pf29Arp population density on the wheat roots in the in vitro confrontation assay. Bacterial density on roots alone (●), on roots early colonized with *Gaeumannomyces graminis* var. *tritici* IV-26/00 (■), or on necrotic roots (▲) was monitored by dilution plating. Results were expressed in CFU per centimeter of roots as means of three biological replicates each containing two technical replicates. Differences between treatments were analyzed by analysis of variance and were significant at $P = 0.05$.

Fig. 6. Pf29Arp differentially expressed genomic regions during tripartite interactions. **A**, Venn diagram showing the number of differentially expressed genomic regions during specific interaction with Pf29Arp. Three ellipses represented the different comparative contrasts: early *G. graminis* var. *tritici*-colonized roots (E) versus healthy roots (H), necrotic roots (N) versus H, and N versus E. The overlapped sectors show the number of genes expressed jointly among various phases. **B**, Genomic regions differentially expressed during Pf29Arp specific interaction were separated into two groups according to whether they were significantly ($P \leq 0.01$) upregulated (white bars) or downregulated (gray bars).

H and N versus E, with 7 genes similarly regulated. This last intersection corresponded to genes differentially regulated on early *G. graminis* var. *tritici*-colonized roots.

Selection and identification of differentially expressed clones.

In total, 114 genomic regions were selected according to their significant fold change in gene expression ($\log_2$ transcript ratio ≤ -1 and ≥ 1) and then sequenced. DNA sequences were compared with databases using the BLASTX algorithm.

Among these 114 genomics regions, 66 were represented once, 16 twice, 4 three times, and 1 four times. Redundant genomic regions were similarly regulated, confirming the reliability of the hybridization data. In total, 169 open reading frames (ORF) were identified, indicating that 33% of the genomic regions contained more than one transcript. Two genomic regions shared no significant homology (*E* value $> 1.10^{-4}$) with sequences in database (clone number 9N22 and 38C24).

Genes were grouped according to their expression pattern in the three conditions (Table 1). Genomic regions which con-

Table 1. Genes of *Pseudomonas fluorescens* Pf29Arp with altered expression between healthy, early colonized, and necrotic roots^a

Clone ^b	Closest homologous gene ^c	Funct. ^d	Gene acc. no. ^c	Locus tag ^c	Organism ^c	Bit score ^e	E value ^e	Evs H ^e	Nvs H ^e	Nvs E ^e	P value
Downregulated^f											
31C6	Putative tricarboxylate transport protein, <i>tctC</i>	1	AAY90723	PFL_1439	<i>P. fluorescens</i> Pf-5	479	2.E-133	-1.0	-1.4	-	6.E-04
12I9	ATP/GTP binding protein, <i>nirQ</i>	2	AAG34380	-	<i>P. fluorescens</i> C7R12	385	2.E-105	-0.7	-1.2	-	3.E-04
19N17	Translation elongation factor Tu, <i>tuf</i>	7	AAY94789	PFL_5584	<i>P. fluorescens</i> Pf-5	305	1.E-81	-1.2	-1.1	-	1.E-03
38D19	RNA polymerase factor sigma-54, <i>rpoN</i>	8	ABA72600	Pf101_0857	<i>P. fluorescens</i> Pf0-1	622	2.E-176	-1.2	-1.1	-	9.E-07
36C1	TonB-dependent siderophore receptor	10	AAY90219	PFL_0932	<i>P. fluorescens</i> Pf-5	682	0.0	-1.1	-1.3	-	5.E-04
27O22	Hypothetical protein	11	ABA72309	Pf101_0565	<i>P. fluorescens</i> Pf0-1	77	1.E-12	-1.0	-1.0	-	2.E-08
11I10	Hypothetical protein	11	CAE12620	plu0325	<i>Photobacterium luminescens</i>	67	3.E-09	-0.9	-1.3	-	7.E-06
Upregulated^g											
25P10	Imidazole glycerol phosphate synthase subunit, <i>hisF</i>	1	ABA72071	Pf101_0327	<i>P. fluorescens</i> Pf0-1	358	2.E-97	1.1	1.3	-	7.E-09
19J20	Coniferyl aldehyde dehydrogenase, <i>calB</i>	1	AAY95055	PFL_5865	<i>P. fluorescens</i> Pf-5	461	9.E-128	1.1	0.6	-	6.E-05
35I15	L-sorbose dehydrogenase	1	AZ36452	PSPPH_1743	<i>P. syringae</i> pv. <i>phaseolicola</i>	219	2.E-55	1.2	1.2	-	6.E-08
18H9	Transcriptional regulator, LysR family, <i>trpI</i>	1, 8	ABX35598	Daci_2960	<i>Delftia acidovorans</i> SPH-1	289	3.E-76	0.8	1.1	-	9.E-09
18F6	Amino acid ABC transporter	1, 10	ABA74830	Pf101_3092	<i>P. fluorescens</i> Pf0-1	110	8.E-23	1.1	0.8	-	2.E-06
13O6	Hypothetical protein	3	ABA73374	Pf101_1631	<i>P. fluorescens</i> Pf0-1	231	5.E-59	0.5	1.1	-	1.E-05
17C21	Hydrogen cyanide synthase, <i>hcnC</i>	4	ABA75252	PFL_2579	<i>P. fluorescens</i> Pf-5	100	1.E-19	1.2	1.3	-	4.E-08
9G10	Alkyl hydroperoxide reductase subunit F, <i>ahpF</i>	5	AAY92663	PFL_3394	<i>P. fluorescens</i> Pf-5	315	4.E-84	0.7	1.0	-	9.E-08
9G10	Alkyl hydroperoxide reductase subunit C, <i>ahpC</i>	5	AAY92664	PFL_3395	<i>P. fluorescens</i> Pf-5	234	7.E-60	0.7	1.0	-	9.E-08
6D17	Chaperone protein, <i>hscC</i>	5	AAY94451	PFL_5232	<i>P. fluorescens</i> Pf-5	139	3.E-31	1.4	1.0	-	1.E-06
24P2	Heat shock protein, <i>dnaJ</i>	5	ABA72507	Pf101_0764	<i>P. fluorescens</i> Pf0-1	418	2.E-115	1.2	1.5	-	7.E-09
6F3	Heavy metal sensor signal transduction	5, 9	ABA76401	Pf101_4664	<i>P. fluorescens</i> Pf0-1	552	1.E-155	1.0	0.8	-	1.E-04
29M23	Pilus assembly protein, <i>pilQ</i>	6	ABA72152	Pf101_0408	<i>P. fluorescens</i> Pf0-1	274	5.E-72	0.8	1.2	-	3.E-04
29M23	Pilus assembly protein, <i>pilO</i>	6	ABA72151	Pf101_0407	<i>P. fluorescens</i> Pf0-1	92	3.E-17	0.8	1.2	-	3.E-04
32C5	Chromosome segregation SMC protein	6	AAY91178	PFL_1891	<i>P. fluorescens</i> Pf-5	376	1.E-102	1.0	1.2	-	2.E-09
6L13	Sensory box/GGDEF family protein	9	ABP79877	PST_2218	<i>P. stutzeri</i> A1501	406	2.E-111	0.4	1.0	-	2.E-07
33K8	Sensory box/GGDEF domain/EAL domain protein	9	ABA75823	PFL_4322	<i>P. fluorescens</i> Pf-5	511	2.E-143	1.0	1.1	-	3.E-09
14B23	Sensory box histidine kinase	9	AAY94834	PFL_5641	<i>P. fluorescens</i> Pf-5	350	3.E-95	1.0	1.4	-	9.E-08
14F7	TonB-dependent siderophore receptor	10	ABZ00648	PputGB1_4761	<i>P. putida</i> GB-1	152	9.E-36	1.2	1.2	-	2.E-06
9L15	Hypothetical protein	11	ABA75715	Pf101_3978	<i>P. fluorescens</i> Pf0-1	395	3.E-108	0.9	1.4	-	6.E-05
3M22	Hypothetical protein	11	ABA76521	Pf101_4784	<i>P. fluorescens</i> Pf0-1	623	1.E-176	1.0	1.1	-	1.E-09
Down, change^h											
32O8	RNA polymerase factor sigma-70, <i>rpoD</i>	8	CAJ29299	-	<i>Pseudomonas</i> sp. OX1	325	2.E-87	-0.4	-1.1	-0.7	2.E-07
Up, changeⁱ											
7K20	Phosphoglyceromutase, <i>gpmA</i>	1	AAY95780	PFL_0371	<i>P. fluorescens</i> Pf-5	374	5.E-102	0.7	1.1	0.4	6.E-08
6H11	Glucose-6-phosphate 1-dehydrogenase, <i>zmf</i>	1	ABA76100	Pf101_4363	<i>P. fluorescens</i> Pf0-1	279	2.E-73	1.2	1.5	0.7	7.E-06

(continued on next page)

^a Genomic regions containing more than one open reading frame (ORF) were discarded for subsequent analysis except for genes which expression was validated by quantitative reverse-transcription polymerase chain reaction (*hcnC*, *rraA*, PputGB1_4761), ORF organized in operon (*pilOQ*, PFL_2600-Pf101_3324, *dinF*-Pf101_0629, *potHA*, Pf101_0106-0107), and one pair with the same biological function (*ahpF*, *ahpC*).

^b Clone number referred to the genomic library number.

^c Best BlastX hit.

^d Genes were classed according to their expression ratios in the three conditions: healthy roots (H), early *G. graminis* var. *tritici*-colonized roots (E), and necrotic roots (N). For each condition, genes were classed by functional categories: 1, carbon-nitrogen metabolism; 2, denitrification pathway; 3, nucleotide-nucleic acid metabolism; 4, secondary metabolism; 5, stress response; 6, motility and cell biogenesis; 7, translation; 8, transcriptional regulator; 9, signaling; and 10, transport.

^e Expression ratio ($\log_2$ fold change $-1 < \text{or} > 1$) of the three contrasts (E versus H, N versus H, and N versus E) is given for genes statistically differentially regulated according to the *F* test ($P < 0.01$); - = genes nonstatistically differentially regulated.

^f Genes downregulated on roots infected with *G. graminis* var. *tritici*.

^g Genes upregulated on roots infected with *G. graminis* var. *tritici*.

^h Genes downregulated on roots infected with *G. graminis* var. *tritici* with highest fold-change on necrotic roots.

ⁱ Genes upregulated on roots infected with *G. graminis* var. *tritici* with highest fold-change on necrotic roots.

^j Gene upregulated on early *G. graminis* var. *tritici* colonized roots.

^k Genes downregulated on necrotic roots.

tained more than one ORF were discarded for subsequent analysis (Supplementary Table S1) except for genes for which expression was validated by quantitative reverse-transcriptase (Q-RT)-PCR (*hcnC*, *rraA*, PputGB1_4761), ORF organized in operon (*pilO/pilQ*, PFL_2600/Pfl01_3324, *dinF*/Pfl01_0629, *potH/potA*, Pfl01_0106/Pfl01_0107), and one pair with the same biological function (*ahpF*, *ahpC*). For most groups, the presence of the fungal pathogen on roots clearly altered Pf29Arp transcript abundance, with a most pronounced effect for necrotic roots.

Many bacterial genes with predicted functions related to nutrient acquisition, including amino acid (e.g., *thrC* encoding

a threonine synthase), carbohydrate (e.g., *gpmA* encoding a phosphoglyceromutase) and tricarboxylate (e.g., PFL_2499 encoding a citrate transporter) transport and metabolism, were differentially expressed on E and N roots compared with H roots. In parallel, global regulators of carbon and nitrogen sources, such as *gbdR* encoding a transcriptional regulator required for glycine betaine catabolism and *cbrB* encoding the regulator of the two-component system CbrAB involved in several pathway regulation in response to C:N ratio, were progressively induced during the development of *G. graminis* var. *tritici* pathogenesis (H < E < N). A gene involved in iron

Table 1. continued from preceding page

Clone ^b	Closest homologous gene ^c	Funct. ^d	Gene acc. no. ^c	Locus tag ^c	Organism ^c	Bit score ^e	E value ^c	Evs H ^e	Nvs H ^e	Nvs E ^e	P value
Up, change ⁱ (continued)											
19P5	DNA-binding response regulator, <i>cbrB</i>	1, 8	AAAY35889	Psyr_0831	<i>P. syringae</i> pv. <i>syringae</i> B728a	321	4.E-86	1.0	1.3	0.6	6.E-08
17P20	Transcriptional activator, AraC family, <i>ftaA</i>	1, 8	ABA73805	Pfl01_2062	<i>P. fluorescens</i> Pf0-1	328	5.E-88	0.5	1.1	0.7	8.E-09
36P5	Transcriptional Regulator, AraC family, <i>gbdR</i>	1, 8	ABA76973	Pfl01_5236	<i>P. fluorescens</i> Pf0-1	390	6.E-107	0.8	1.2	0.4	2.E-10
10M1	DNA primase, <i>dnaG</i>	3	ABA76885	Pfl01_5148	<i>P. fluorescens</i> Pf0-1	392	2.E-107	0.7	1.4	0.7	5.E-06
19D24	Ribonuclease activity regulator protein, <i>rraA</i>	3	AAAY91159	PFL_1871	<i>P. fluorescens</i> Pf-5	113	2.E-23	0.9	1.3	0.3	5.E-10
35K6	DNA polymerase III subunits gamma and tau, <i>dnaX</i>	3	ABA73548	Pfl01_1805	<i>P. fluorescens</i> Pf0-1	617	4.E-175	0.9	1.1	0.4	5.E-10
36O21	Ribose-phosphate pyrophosphokinase, <i>prs</i>	3	AAAY96268	PFL_5165	<i>P. fluorescens</i> Pf-5	310	1.E-82	1.0	1.3	0.5	3.E-11
33G15	RNA 3'-terminal-phosphate cyclase, <i>rtcA</i>	3	ABP83956	Pmen_1191	<i>P. mendocina</i> ymp	120	4.E-52	1.4	1.9	0.7	3.E-09
35G3	Amino acid adenylation	4	ABA73590	Pfl01_1847	<i>P. fluorescens</i> Pf0-1	293	1.E-77	0.8	1.1	0.4	9.E-10
13O4	Putative catalase	5	AAAY93210	PFL_3946	<i>P. fluorescens</i> Pf-5	383	8.E-105	0.4	1.2	0.6	1.E-07
17L21	Cytochrome c oxidase, subunit II, <i>coxB</i>	5	ABA71823	Pfl01_0079	<i>P. fluorescens</i> Pf0-1	504	3.E-141	0.6	1.1	0.6	4.E-09
20C8	DnaJ domain protein, putative	5	AAAY94452	PFL_5233	<i>P. fluorescens</i> Pf-5	80	2.E-13	1.0	1.3	0.6	6.E-10
31G7	Multidrug extrusion superfamily, <i>dinF</i>	5, 10	AAAY96087	PFL_0680	<i>P. fluorescens</i> Pf-5	141	6.E-32	0.9	1.3	0.5	3.E-11
10J1	Glycosyl transferase, family 2	6	ABJ81477	Acid_0467	<i>Solibacter usitatus</i> Ellin6076	113	2.E-23	0.5	1.0	0.4	7.E-07
36A21	Periplasmic beta-glucosidase, <i>bglX</i>	6	AAAY90636	PFL_1351	<i>P. fluorescens</i> Pf-5	788	0.0	0.9	1.2	0.5	6.E-10
20P19	S-adenosylmethionine:tRNA, <i>queA</i>	7	AAT96042	-	<i>P. viridiflava</i>	205	5.E-51	0.8	1.5	0.9	7.E-11
3A22	Transcriptional regulator LuxR family	8	ABA74200	Pfl01_2459	<i>P. fluorescens</i> Pf0-1	163	1.E-38	0.9	1.2	0.6	6.E-08
10N19	Transcriptional Regulator, AraC family	8	ABA74612	Pfl01_2871	<i>P. fluorescens</i> Pf0-1	473	7.E-132	1.4	1.8	1.0	3.E-08
37I4	Putative diguanylate cyclase/phosphodiesterase	9	ABA76992	Pfl01_5255	<i>P. fluorescens</i> Pf0-1	516	1.E-144	0.7	1.0	0.4	6.E-10
20K1	Hemolysin activator protein, HlyB family	10	AAAY90834	PFL_1551	<i>P. fluorescens</i> Pf-5	545	2.E-153	0.6	1.0	0.4	9.E-10
36O23	Rhs element Vgr protein	10	ABA75416	Pfl01_3678	<i>P. fluorescens</i> Pf0-1	237	8.E-61	0.7	1.1	0.4	9.E-11
19C13	Binding-protein-dependent transport systems, <i>potH</i>	10	ABA72789	Pfl01_1046	<i>P. fluorescens</i> Pf0-1	179	5.E-42	1.0	1.6	0.6	2.E-08
19C13	Spermidine/putrescine ABC transporter, <i>potA</i>	10	ABA72790	Pfl01_1047	<i>P. fluorescens</i> Pf0-1	297	7.E-79	1.0	1.6	0.6	2.E-08
37N1	Outer membrane ferric siderophore receptor, putative	10	AAN68936	PP3330	<i>P. putida</i> KT2440	268	3.E-70	2.1	2.5	1.1	3.E-11
9O11	Hypothetical protein	11	ABA76031	Pfl01_4294	<i>P. fluorescens</i> Pf0-1	145	4.E-33	0.6	1.1	0.5	1.E-07
31E24	Hypothetical protein	11	AAO58016	PSPTO_4570	<i>P. syringae</i> pv. <i>tomato</i> DC3000	115	5.E-24	0.7	1.4	0.6	2.E-09
34E21	Hypothetical protein	11	AAAY91873	PFL_2600	<i>P. fluorescens</i> Pf-5	102	3.E-20	0.8	1.1	0.6	1.E-07
34E21	Hypothetical protein	11	ABA75061	Pfl01_3324	<i>P. fluorescens</i> Pf0-1	117	1.E-24	0.8	1.1	0.6	1.E-07
31G7	Hypothetical protein	11	ABA72373	Pfl01_0629	<i>P. fluorescens</i> Pf0-1	405	4.E-51	0.9	1.3	0.5	3.E-11
15A1	Hypothetical protein	11	ABM33693	Aave_3129	<i>Acidovorax avenae</i> AAC00-1	106	3.E-21	0.9	1.4	0.5	9.E-09
5B6	Hypothetical protein	11	ABA71850	Pfl01_0106	<i>P. fluorescens</i> Pf0-1	469	1.E-130	1.8	2.4	1.2	9.E-10
5B6	Hypothetical protein	11	ABA71851	Pfl01_0107	<i>P. fluorescens</i> Pf0-1	87	2.E-15	1.8	2.4	1.2	9.E-10
5K9	Hypothetical protein	11	ABA71785	Pfl01_0041	<i>P. fluorescens</i> Pf0-1	442	2.E-122	2.1	0.5	0.3	2.E-07
Up, early ^j											
3P9	Toluene tolerance protein, putative, <i>tgg8</i>	5	AAAY35605	Psyr_0535	<i>P. syringae</i> pv. <i>syringae</i> B728a	276	2.E-72	1.6	-0.3	-	1.E-03
Down, necrotic ^k											
25K13	Aldose 1-epimerase	1	ABA76102	Pfl01_4365	<i>P. fluorescens</i> Pf0-1	536	1.E-150	-	-1.1	-1.0	1.E-06
33B13	Major facilitator superfamily MFS_1	5, 10	AAAY93662	PFL_4411	<i>P. fluorescens</i> Pf-5	343	1.E-92	-	-1.0	-1.1	1.E-06
8P10	Hypothetical protein	11	ABA76950	Pfl01_5213	<i>P. fluorescens</i> Pf0-1	243	1.E-62	-	-1.3	-0.8	5.E-06
12B17	Hypothetical protein	11	ABA77411	Pfl01_5675	<i>P. fluorescens</i> Pf0-1	396	1.E-108	-	-1.2	-1.1	6.E-09
8I2	Threonine synthase, <i>thrC</i>	1	AAAY90391	PFL_1104	<i>P. fluorescens</i> Pf-5	291	7.E-77	-	-1.0	-	5.E-04
34O11	Phosphate starvation-inducible protein, <i>psiF</i>	9	AAAY96027	PFL_0620	<i>P. fluorescens</i> Pf-5	151	6.E-35	-	-1.3	-	1.E-03

acquisition (PputGB1_4761 encoding a TonB-dependent siderophore receptor) was upregulated on E and N, whereas a gene of the denitrification process (*nirQ* encoding a putative ATP-binding protein) was positively regulated on H roots.

Another relevant result was that bacterial genes related to stress (e.g., PFL_3946 encoding a putative catalase) and detoxification processes (e.g., *dinF* and *tig8* encoding DNA-damage-inducible protein F and a putative toluene tolerance protein, respectively) were induced in E and N compared with H. A number of chemotaxis (e.g., PSPA7_2644 encoding a putative chemotaxis transducer) and cell biogenesis-related genes (e.g., *bgIX* encoding a periplasmic beta-glucosidase) were also upregulated in E and N. The same pattern of regulation was observed for genes encoding secreted proteins (e.g., PFL_1551 and Pfl01_3678 encoding a hemolysin activator protein belonging to the T5SS TpsB family and a putative type VI secretion system [T6SS] effector, respectively) and in the modulation of RNA abundance (e.g., *rraA* encoding a ribonuclease activity regulator protein). Finally, the gene *hcnC* encoding a putative amino-acid oxidase involved in hydrogen cyanide (HCN) synthesis was upregulated by the fungal presence.

Validation of selected gene expression.

To validate the results obtained with the Pf29Arp DNA microarray, Q-RT-PCR was performed on 11 genes differentially regulated and which were representative of each gene class (except for class 7, 8, 9, and 11). This included the following genes: *thrC*, *bgIX*, *nirQ*, PFL_3946, *dinF*, Psyr_0535, PputGB1_4761, PFL_1551, *rraA*, and *hcnC* (Supplementary Table S2).

The analysis showed that the majority of transcripts (*bgIX*, PFL_3946, PFL_1551, PputGB1_4761, *rraA*, and *hcnC*) exhibited the same expression ratios as observed with the microarrays (Table 2). However, some genes that exhibited small fold changes on microarray in a specific condition (e.g., *dinF*, Pfl01_3678, and Psyr_0535) were apparently not differentially expressed, and genes not regulated in the microarray at a specific stage (e.g., *thrC*, *dinF*, and *nirQ*) were differentially expressed as mean by Q-RT-PCR.

DISCUSSION

In this work, we have tracked alterations of bacterial transcripts during the establishment of Pf29Arp populations at the surface of wheat roots infected with *G. graminis* var. *tritici*. First, pathogenesis and bacterial colonization were monitored

in tripartite gnotobiotic experiments and compared with bipartite experiments. In the absence of bacterial inoculation, *G. graminis* var. *tritici* mycelium first grew mainly ectotrophically on roots (stage E); then, the mycelium reached the stele and the necrosis largely extended (stage N). Only inoculation of Pf29Arp on early *G. graminis* var. *tritici*-colonized roots led to a drop in incidence and severity of take-all disease after 48 h of interaction compared with the control without bacterial inoculation. This effect could be explained by different direct and indirect mechanisms such as competition for niches and nutrient between Pf29Arp and *G. graminis* var. *tritici* outside the plant, antibiosis or fungistasis toward *G. graminis* var. *tritici*, and induced plant resistance (Haas and Defago 2005). Such niche competition was already reported between *F. oxysporum* and biocontrol pseudomonads on tomato roots at the intercellular junctions (Bolwerk et al. 2003). In contrast, when necrosis or deep root colonization was first developed (N), no biocontrol activity could affect the protected mycelium inside roots.

Although the same population density of Pf29Arp was reached at 48 h in each of the three treatments (H, E, and N), differences in early colonization patterns were observed. On healthy roots, population density reached 5×10^6 CFU cm⁻¹ of roots at 24 h and was maintained up to 96 h. Unexpectedly, after the onset of necrosis, Pf29Arp population density dramatically and rapidly dropped. Stressful environment conditions, such as accumulation of phenolics compounds on necrotic roots (Rengel et al. 1994), could explain this rapid bacterial cell death. Necrosis could also have changed root surface physical properties and avoided bacterial adhesion to root tissues. On early *G. graminis* var. *tritici*-colonized roots, a four times less increase in bacterial multiplication rate was observed, which could be explained by competition for nutrients and space between Pf29Arp and *G. graminis* var. *tritici*.

Here, bacterial and fungal fitness were measured as Pf29Arp growth rate and take-all severity and incidence, respectively. On early *G. graminis* var. *tritici*-colonized roots, the interspecific relationship was negative for both microbial partners compared with their respective growth and infectivity on roots without the other partner. Therefore, competitive interactions between Pf29Arp and *G. graminis* var. *tritici* have occurred. On necrotic roots, the interaction was neutralistic because neither negative nor beneficial effects were measured for the fungus and Pf29Arp. Usually, biocontrol strains are described as antagonistic or parasitic toward fungi missing mutualistic and neutral rhizobacteria occurrence in the same pathosystem as described by Samiuguet and associates (1992). Because commensal and

Table 2. Relative expression levels of *Pseudomonas fluorescens* Pf29Arp genes by the microarray approach versus the quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) approach^a

Genes	Putative function of protein	E versus H		N versus H		N versus E	
		Microarray	Q-RT-PCR ^b	Microarray	Q-RT-PCR ^b	Microarray	Q-RT-PCR ^b
<i>thrC</i>	Threonine synthase	–	–0.85	–0.99	–0.71	–	0.12
<i>bgIX</i>	Periplasmic beta-glucosidase	0.94	0.74	1.24	1.70	0.49	1.04
<i>nirQ</i>	ATP/GTP binding protein, NirQ	–0.71	–1.33	–0.60	–0.38	–	0.69
PFL_3946	Catalase, putative	0.44	0.24	1.18	0.89	0.57	0.66
<i>dinF</i>	DNA-damage-inducible protein F	0.94	0.08	1.33	0.72	0.51	0.91
Psyr_0535	Toluene tolerance protein putative	1.58	0.84	–0.30	0.49	–	–0.58
PputGB1_4761	TonB-dependent siderophore receptor	1.20	0.51	1.18	0.83	–	0.36
Pfl01_3678	Rhs element Vgr protein	0.72	0.17	1.11	1.20	0.42	1.00
PFL_1551	Hemolysin activator protein, HlyB family	0.61	0.75	1.02	0.94	0.41	0.07
<i>rraA</i>	Ribonuclease activity regulator protein	0.86	0.44	1.30	1.08	0.31	0.60
<i>hcnC</i>	Hydrogen cyanide synthase	1.21	1.43	1.28	2.10	–	0.42

^a Data came from measurements performed by real-time PCR on Pf29Arp cDNAs obtained from healthy (H), *G. graminis* var. *tritici* early-colonized (E), and necrotic roots (N). Three pairwise comparisons (E versus H, N versus H, and N versus E) were made. The expression level of each gene of interest was calculated by dividing the quantities for each sample by the appropriate geNorm normalization factor.

^b Each value is the mean of three biological replicates and two technical replicates. Data in bold highlight significant changes in expression level (interval confidence, $P = 0.05$); – = genes nonstatistically regulated on microarray.

mutualistic interactions were previously observed during in vitro co-cultures of Pf29Arp with *G. graminis* var. *tritici* (Barret et al. 2009) and with the ectomycorrhizal fungus *Laccaria bicolor* S238N (Deveau et al. 2007), respectively, our results highlight that the same biocontrol rhizobacteria can differentially interact with a filamentous fungus along its life cycle.

Roots infected with *G. graminis* var. *tritici* strongly modified Pf29Arp transcriptome with 100- to 400-fold more genomic regions differentially expressed compared with *G. graminis* var. *tritici* alone (Barret et al. 2009). In total, 27 common genomic regions were significantly regulated in both the *G. graminis* var. *tritici* hyphosphere and roots infected with *G. graminis* var. *tritici*. However, only one gene (PputGB1_4761) had a twofold change-expression ratio. The low overlap between these outcomes could be explained by differences in experimental conditions. Indeed, direct influence of *G. graminis* var. *tritici* on Pf29Arp transcript abundance was studied before cell-to-cell contact up to contact on a complete media (Barret et al. 2009).

Two main expression patterns of sequenced clones were identified. The first pattern fitted with genes regulated by the fungal presence on roots compared with healthy roots. For example, *nirQ*, which encodes a putative ATP-binding protein, was downregulated during the two *G. graminis* var. *tritici* infection stages when compared with healthy roots. NirQ is involved in regulation of the nitric oxide (NO) reduction pathway in *P. stutzeri* (Jungst and Zumft 1992). NO is generated and reduced by bacterial denitrification, a process in which bacteria utilize oxidized nitrogen as electron acceptors when oxygen concentration is limited. Denitrification may constitute a significant advantage for root colonization because denitrifying bacteria predominantly occur near or inside roots (Philippot et al. 1995; Ghigliione et al. 2000). Here, we showed that a homologous genes was upregulated on healthy roots and downregulated in the presence of *G. graminis* var. *tritici* (E and N), meaning that understanding the entire process of root colonization by biocontrol bacteria must take into account the presence of pathogens.

The gene *hcnC*, belonging to the *hcnABC* operon encoding cyanide synthase (Laville et al. 1998), was also upregulated on *G. graminis* var. *tritici* infected roots. In *P. fluorescens*, HCN synthesis accounts for a part of the biocontrol capacity against suppression of *Thielaviopsis basicola* (Voisard et al. 1989) and *G. graminis* var. *tritici* (Sarniguet et al. 1992; Blumer and Haas 2000). Similarly, the presence of *Rhizoctonia solani* in the bean rhizosphere increases *hcnA* expression in the biocontrol strain *P. fluorescens* CHA0 (Jamali et al. 2009). However, HCN synthesis by Pf29Arp was not detected in vitro on glycine medium (data not shown). Because HCN synthesis could be effective only under low oxygen pressure in *P. aeruginosa* (Cody et al. 2009), production of this metabolite on *G. graminis* var. *tritici*-colonized roots remains to be assessed.

On roots colonized with *G. graminis* var. *tritici*, an ORF sharing 82% identity at the amino acid level with a hemolysin activator protein of *P. fluorescens* Pf-5 (PFL_1551) was identified. This gene belonged to the TpsB transporter family of the two-partner secretion pathway (TPS) (Jacob-Dubuisson et al. 2001). TPS systems are encoded by two genes generically designated *tpsA*, encoding the secreted protein and *tpsB*, encoding a dedicated transporter, which are frequently located in an operon (Jacob-Dubuisson et al. 2004). The gene immediately downstream, PFL_1551 in *P. fluorescens* Pf29Arp, shared 53% identity at the amino acid level with a filamentous hemagglutinin protein (PFL_1552) of *P. fluorescens* Pf-5 that is the TpsA-secreted protein. Haemagglutinin facilitates the attachment of bacteria to mammalian (Locht et al. 1993) and vegetal (Rojas et

al. 2002) host-cells; therefore, PFL_1551 could also be involved in this trait.

The second expression pattern of Pf29Arp-sequenced clones contained genes highly regulated on necrotic roots when compared with early-colonized roots. For example, a significant induction for genes involved in C and N metabolism was detected between necrotic roots and roots early colonized with *G. graminis* var. *tritici*, which could suggest either an increase in nutrient availability due to leakage from damaged root tissues (Curl and Truelove 1986) or a nutrient limitation on this environment. For example, *gbdR*, which encodes the regulator of glycine betaine catabolic genes that is activated by glycine betaine accumulation (Wargo et al. 2008), is induced on necrotic roots. Similarly, induction of *cbrB* encoding the regulator of the two-component system CbrAB which controls the expression of numerous catabolic pathways in response to changing intracellular C:N ratios in *Pseudomonas* spp. (Nishijyo et al. 2001; Zhang and Rainey 2008) could reflect a shift in nutrient availability.

Bacterial genes with predicted functions related to oxidative stress (gene PFL_3946 encoding a catalase, *ahpF*, *ahpC*) and detoxification process (*dinF*) were upregulated on *G. graminis* var. *tritici*-colonized roots with a significant increase in expression ratio on necrotic roots. The progressive induction of bacterial stress-responsive genes during *G. graminis* var. *tritici* development was probably linked to continually increasing levels of plant defense mechanisms against this fungus. Indeed, the first step of the defense mechanism in plants involves a rapid accumulation of phenols at the infection site (Nicholson and Hammerschmidt 1992). Following this, phenol oxidation leads to browning of tissues (Hammerschmidt 2005) and the formation of quinones and free radicals that have antimicrobial activities (Appel 1992). In agreements with these observations, Sari and associates (2008) have shown that the amount of phenolic compounds and oxidase enzymes in wheat roots treated with *G. graminis* var. *tritici* and the biocontrol strain *P. fluorescens* CHA0 increases progressively until 6 days after pathogen inoculation. Although this could explain the upregulation of bacterial genes involved in oxidative stress and detoxification process, we cannot exclude the possibility that compounds produced by the fungus itself could also activate some of these bacterial stress-responsive genes, as already observed in Pf29Arp-*G. graminis* var. *tritici* co-cultures (Barret et al. 2009). Even when colonizing healthy roots, Pf29Arp has to develop protection mechanisms because genes encoding glutathione S-transferase and multidrug resistance efflux pump were induced at this stage. Clearly, stress adaptation is an important trait for colonization of roots infected or not with *G. graminis* var. *tritici*.

Among this second expression pattern, some Pf29Arp genes were specifically induced on necrotic roots. For example, *rraA* was specifically upregulated on necrotic roots. In *Escherichia coli*, the protein RraA interacts with RNase E and inhibits its endoribonuclease action, leading to global change in RNA abundance (Lee et al. 2003). Furthermore, instead of simply acting as general inhibitors of RNase E, RraA specifically modulates RNA decay and, therefore, affects the intracellular levels of distinct sets of transcript (Gao et al. 2006). No experimental evidence on the role of RraA in *Pseudomonas* spp. is yet available, but *rraA* is widely distributed among bacteria and plants (Lee et al. 2003) and possesses the same intrinsic activity in other bacterial species (Yeon et al. 2008; Lee et al. 2009).

Similarly, one ORF, which shares 53% identity at the amino acid level with an Rhs element Vgr protein of *P. fluorescens* Pf0-1 (Pfl01_3678), was specifically upregulated on necrotic roots. The predicted protein of Pf29Arp belongs to the VgrG protein family of the type VI effector, as suggested by domains analysis by InterProScan. T6SS is defined by a set of structural

components which may vary in terms of organization and composition between species (Filloux et al. 2008; Pukatzki et al. 2009). In many species, T6SS appears to be a major virulence determinant (Mougous et al. 2006; Pukatzki et al. 2007) whereas, in others, it decreases the infection (Mattinen et al. 2008; Wu et al. 2008). T6SS is also present in nonpathogenic bacteria such as the biocontrol strain *P. fluorescens* Pf-5 (Paulsen et al. 2005). We also have some evidence that Pf29Arp contains at least four T6SS clusters (*unpublished results*). The role of Pf29Arp T6SS and associated effectors in colonization of necrotic roots remains to be assessed.

In conclusion, the two-step infection process of roots by the take-all fungus provided strong support for the concept of pathorhizosphere. Indeed, changes in gene expression of Pf29Arp do not simply reflect the influence of either roots or *G. graminis* var. *tritici* alone. The set of genes triggered on necrotic roots gives evidence of an adaptative response of Pf29Arp to this environment. This phenomenon could explain the role of necrotic roots in the selection of some adapted rhizobacteria after a disease outbreak in wheat fields. However, other regulation levels such as post-transcriptional controls with the Gac/Rsm cascade (Kay et al. 2005) probably occurred in this environment.

MATERIALS AND METHODS

Plant growth assay.

Wheat seeds of *Triticum aestivum* cv. Talent were placed in sterile deionized water, sonicated (40 Hz, 2 min 30 s), surface sterilized for 5 min with a 2.5% hypochlorite solution, and rinsed five times with sterile deionized water for 5 min. Then, seeds were soaked in 42°C preheated, deionized water for 10 min. Five seeds per plate were placed on LPGA medium (yeast extract at 5 g liter⁻¹ [Difco, Franklin Lakes, NJ, U.S.A.], peptone at 5 g liter⁻¹ from meat [Merck, Darmstadt, Germany], glucose at 5 g liter⁻¹ [Sigma-Aldrich, St. Louis], and agar at 15 g liter⁻¹ [VWR, Val de Fontenay, France]) and a 50- μ l drop of sterilized water was poured every 24 h on each seed. Germination and absence of microbial contamination were assessed after 2 days of incubation at 27°C. Then, sterile tubes were filled with 10 ml of MS media (Sigma-Aldrich) closed with a pierced closure. Germinated seed were sustained in the closure with roots descending into the liquid media. To preserve a gnotobiotical environment, the system was covered with larger sterile tubes and then was incubated at 15°C with a cycle of alternating 14 h of light and 10 h of darkness (irradiance 4 Wm⁻²). After 5 days, seedlings were carefully collected and roots were rinsed with sterile deionized water. Each seedling was laid at the border of a 9-cm petri dish containing water agar (30 g liter⁻¹). A hole was previously made at the border and also in the lid of the dish to let the roots grow gnotobiotically inside and to let the leaf outside (Fig. 1). Each dish was closed with Parafilm M (Pechiney, Chicago), surrounded with an aluminum sheet, and placed inside a larger 15-cm sterile dish to retain a sterile opaque environment. The plates were placed vertically in an incubator at 15°C with the leaf orientated in front and a cycle of alternating 14 h of light and 10 h of darkness.

Fungal and bacterial root inoculations.

The soilborne pathogenic fungus *G. graminis* var. *tritici* IV-26/00 (Willcoquet et al. 2008) was maintained on potato dextrose agar (PDA) (Merck) at 20°C for a week. The fungal inoculum consisted of 8-mm³ plugs (2 by 2 by 2 mm) cut from the colony edge of the growing filamentous *G. graminis* var. *tritici*. For each sterile seedling, three plugs were placed on the three seminal roots at 3 cm below the seed and incubated at 15°C. Then, plugs were removed after 48 h, allowing *G. graminis* var. *tritici* mycelium to grow along the root surface.

P. fluorescens Pf29Arp (Chapon et al. 2002) was first grown on King's B medium (*Pseudomonas* F agar at 35 g liter⁻¹ and 1% glycerol) for two days at 27°C. Then, one colony was picked up and suspended in 10 ml of LPG overnight at 27°C under constant agitation (150 rpm). The bacteria were centrifuged at 4,500 $\times$ g for 5 min and the pellet was washed once and resuspended in deionized water to obtain a suspension with an optical density at 600 nm = 0.25 (10⁸ CFU ml⁻¹). Fungal plugs were removed from the roots and two drops of 5 μ l (10⁶ cells) were immediately deposited side by side on each root at 3 cm from the seed, right through the deposit of the fungal plug. Seedlings were then incubated at 15°C.

Influence of roots and *G. graminis* var. *tritici* was followed at two stages of the *G. graminis* var. *tritici* infection process. Indeed, *G. graminis* var. *tritici* first grew on the wheat root surface as melanized ectotrophic "runner" hyphae; then, hyaline hyphae emerged and penetrated the root cortex and ultimately the stele, which led to necrotic roots (Freeman and Ward 2004). Therefore, we built up a bioassay in which the biocontrol strain Pf29Arp was inoculated on three distinct wheat roots environments: healthy (H); early *G. graminis* var. *tritici* colonized (E), which correspond to the first stage of the *G. graminis* var. *tritici* infection process with a 2-day-old *G. graminis* var. *tritici* inoculation; and necrotic roots (N), with a 5-day-old *G. graminis* var. *tritici* inoculation. For the three conditions, Pf29Arp was inoculated at day 12 on the in vitro growing root; therefore, 2 days after *G. graminis* var. *tritici* inoculation for E and 5 days after *G. graminis* var. *tritici* inoculation for N (Fig. 2). Controls for Pf29Arp effect and root disease assessments consisted of the same three conditions (H, E, and N) without any Pf29Arp inoculation.

Root disease quantification and observation.

Disease was assessed for E and N conditions in the presence or absence of Pf29Arp inoculation and at 4, 5, and 7 days after *G. graminis* var. *tritici* inoculation. Disease incidence corresponded to the frequency of roots with a dark necrotic symptom (at least 1 mm in length) and disease severity corresponded to the length of dark necrosis. The observations were conducted on each of three roots of nine seedlings for each condition and independently repeat twice.

Fungal growth at the surface of the roots was studied by a binocular microscope and light microscopy of stained mycelium as outlined by Resendes and associates (2001). Each day following *G. graminis* var. *tritici* inoculation, three roots per treatment were sampled and cut into 2-cm-long segments (1 cm at each side of the fungal plug). Segments were fixed overnight in 50% ethanol, transferred to 5% KOH (wt/vol), autoclaved for 15 min at 120°C to clear, rinsed in water, and, finally, placed in the stain at 50°C for 1 h. The stain consisted of 0.1% Chlorazol Black E, 85% lactic acid, and glycerol (1:1:1, vol/vol/vol). The stained segments were mounted and examined under a binocular microscope. Internal invasion of plant tissue by *G. graminis* var. *tritici* was also studied by another method using a modification of the above clearing and staining technique. Segments (2 cm long) were fixed overnight in 50% ethanol, stained with Chlorazol Black E for 5 min, rinsed two times in sterile water, and then placed on a slide. Cross-sections of root segment were done every 1 mm under a binocular microscope ($\times 12$) and segments were mounted and examined by light microscopy. These experiments were independently repeated three times.

Bacterial population assessment.

P. fluorescens Pf29Arp growth was monitored by dilution plating 20 min after bacterial inoculation and then every 24 h during 4 days. For this purpose, root fragments of 8 mm around

Pf29Arp inoculation spot (3 cm below the seed) were cut for each treatment, suspended in 1 ml of sterile water, and sonicated two times (40 Hz, 3 s). The bacterial suspension was 10-fold diluted and 10- μ l aliquots of the dilution (three replicates per dilution) were plated on King B medium. Colonies were counted after incubation at 27°C for 2 days. For each treatment (absence or presence of *G. graminis* var. *tritici* and Pf29Arp), results were expressed in log₁₀ CFU cm⁻¹ of roots as means of three biological replicates (three different plants) with two technical replicates per biological replicate.

To test equalities between experimental treatment for mean necrosis length and mean log₁₀ CFU cm⁻¹ of roots, analysis of variance and Student's test were performed ($P = 0.05$). Equalities between experimental treatments for incidence were assessed by confidence interval of mean differences ($P = 0.05$).

RNA extraction, amplification, and cDNA labeling.

Eighteen root fragments of 8 mm each from six different plants were cut for each independent biological replicates of each treatment (H, E, and N) suspended in 1 ml of RNAlater (Ambion, Austin, TX, U.S.A.) and stored at -20°C. Suspension was sonicated two times (40 Hz, 3 s) and centrifuged (6,000 $\times$ g, 5 min). The pellet was resuspended with 100 μ l of supernatant to avoid loss of bacterial cells and total RNA from *P. fluorescens* Pf29Arp was extracted as described earlier (Barret et al. 2009). RNA yield was determined spectrophotometrically (Agilent, Santa Clara CA, U.S.A.) and RNA quality checked by the Bioanalyzer 2100 (Agilent). Total RNA was amplified through a method described earlier by Moreno-Paz and Parro (2006) based on a random priming approach with the T7 RNA polymerase using the reagents and components of the MessageAmp amplified RNA (aRNA) kit (Ambion). aRNA (5 μ g) was labeled with the ChipShot Indirect Labeling and Clean-Up System (Promega Corp., Madison, WI, U.S.A.), and the CyDye (Cy3/Cy5) Reactive Dye Protocols for Post-Labeling Amino-allyl-cDNA (Amersham Biosciences, Piscataway, NJ, U.S.A.). The efficiency of Cy label incorporation into the cDNA and the quality and amounts of labeled cDNA was verified using a Nanodrop (Agilent).

To assess bias introduced by the amplification system, we hybridized amplified against unamplified RNA extracted from a 48-h-old culture of *P. fluorescens* Pf29Arp on a complete medium (Barret et al. 2009). Two technical replicates corresponding to a dye-swap were performed for this experiment and data were analyzed as described below.

Microarray experiment and data analysis.

Shotgun DNA microarrays were constructed from a PCR-amplified gene library from *P. fluorescens* Pf29Arp as previously described (Barret et al. 2009). The array contained 14,000 PCR products representing approximately 80% of the Pf29Arp genome.

Hybridization was performed on a Ventana Discovery system (Ventana, Tucson, AZ, U.S.A.) for 8 h at 42°C using the OUEST-genopole transcriptomic facilities (IFR 140 GFAS, Rennes, France). Washes were performed manually at room temperature in two successive 1 $\times$ Ribowash solutions (Ventana) followed by a wash in 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature. Fluorescent images of the microarrays, which were generated with a Genepix 4000B scanner, were then analyzed with the Genepix Pro analysis software (v6.0; Molecular Devices, Sunnyvale, CA, U.S.A.).

A loop design was applied for running the microarrays to minimize the variance of contrasts of interest with a convenient number of arrays. Samples (H, E, and N) were hybridized in pairs (E versus H, N versus H, and N versus E) on three different slides. For each pair, three independent biological replicates

and two technical replicates (dye-swap) were performed. Thus, in total, 18 slides were hybridized and compared. Raw data from microarray hybridization, exported from GenePix suites 6.0, was imported into LIMMA (Smyth and Speed 2003) with annotation and spot types. Spots with a negative flag value, a signal-to-noise ratio (SNR) threshold ≤ 2 and an irregular aspect were assigned a weight of 0 in the subsequent analysis. Signal intensities were normalized using a print-tip group Loess normalization (within-array normalization). Statistical values for gene expression of sample pairs were calculated after fitting a mixed-model analysis of variance (model provided by LIMMA) in R Software by using the LIMMA package (Smyth 2004). This statistical analysis permitted us to take into account the negative correlation which exists between technical replicates (dye-swap pairs). Moreover, the method uses empirical Bayes methods to moderate the standard deviation between genes (Milliken and Johnson 1992). The adjusted P value was then assessed for each gene by controlling the false discovery rate using the Benjamini Hochberg method (Benjamini and Hochberg 1995). To investigate which levels of factor differ when the global F test from our model is significant ($P < 0.01$), pairwise comparisons were made by using planned contrasts (E versus H, N versus H, and N versus E). Three lists of differential genes were then obtained with 1% of false positive in each list.

Clones sequencing.

Sequencing was carried out by GATC Biotech (Konstanz, Germany). Sequences were analyzed using Bioedit software, and Blast2go (Conesa et al. 2005) was used for searching for similarities on the *Pseudomonas* genome database (Winsor et al. 2009) and on the nonredundant protein and DNA sequence database at the National Center for Biotechnology Information (Pruitt et al. 2005).

Real-time RT-PCR.

Real-time PCR analyses were performed on some selected differentially expressed genes. The expression stability of five constitutive genes (Supplementary Table S3) was calculated using geNorm (Vandesompele et al. 2002). Two of them (*ppkA* and *quiP*, encoding a serine/threonine protein kinase and a protein related to penicillin acylase, respectively) were chosen for data normalization. This approach relies on the principle that the expression ratio of two ideal control genes is constant in all the samples, independent of the experimental conditions. Genes with the lowest gene expression stability (M) have the most stable expression while the highest M value indicates the least stable expression (Vandesompele et al. 2002). aRNA (500 ng) used to hybridize the array slides was reverse transcribed using the ImProm-I Reverse Transcription System (Promega Corp.). qPCR reactions (25 μ l) containing 2 μ l of cDNA, 300 nM each primer, 1 $\times$ iTaq SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.) were performed on the ABI Prism s7700 sequence detection system. Amplification conditions were initial denaturation at 95°C for 5 min and 40 cycles of 95°C for 30 s and 60°C for 30 s. Standard curve was performed for each gene on four different dilutions of cDNA products. A dissociation curve was constructed at the end of each run in order to detect nonspecific amplifications. The Ct values were transformed to quantities by using standard curves. The expression level of each gene of interest was calculated by dividing the quantities for each sample by the appropriate geNorm normalization factor.

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