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The biocontrol bacterium *Pseudomonas fluorescens* Pf29Arp strain affects the pathogenesis-related gene expression of the take-all fungus *Gaeumannomyces graminis* var. *tritici* on wheat roots

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SUMMARY

The main effects of antagonistic rhizobacteria on plant pathogenic fungi are antibiosis, fungistasis or an indirect constraint through the induction of a plant defence response. To explore different biocontrol mechanisms, an *in vitro* confrontation assay was conducted with the rhizobacterium *Pseudomonas fluorescens* Pf29Arp as a biocontrol agent of the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) on wheat roots. In parallel with the assessment of disease extension, together with the bacterial and fungal root colonization rates, the transcript levels of candidate fungal pathogenicity and plant-induced genes were monitored during the 10-day infection process. The bacterial inoculation of wheat roots with the Pf29Arp strain reduced the development of *Ggt*-induced disease expressed as attack frequency and necrosis length. The growth rates of *Ggt* and Pf29Arp, monitored through quantitative polymerase chain reaction of DNA amounts with a part of the *Ggt 18S rDNA* gene and a specific Pf29Arp strain detection probe, respectively, increased throughout the interactions. Bacterial antagonism and colonization had no significant effect on root colonization by *Ggt*. The expression of fungal and plant genes was quantified *in planta* by quantitative reverse transcription-polymerase chain reaction during the interactions thanks to the design of specific primers and an innovative universal reference system. During the early stages of the tripartite interaction, several of the fungal genes assayed were down-regulated by Pf29Arp, including two laccases, a β-1,3-exoglucanase and a mitogen-activated protein kinase. The plant host glutathione-*S*-transferase gene was induced by *Ggt* alone and up-regulated by Pf29Arp bacteria in interaction with the pathogen. We conclude that Pf29Arp antagonism acts through the alteration of fungal pathogenesis and probably through the activation of host defences.

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INTRODUCTION

The ability of a soil-borne fungus to produce disease on roots (pathogenicity) and its degree of pathogenicity (virulence or aggressiveness) depend, as in the case of other pathogens, on the ability to reach and colonize the root surface (adherence and initial multiplication), the production of extracellular enzymes, toxins and different effectors, which facilitate further tissue invasion, and the bypassing of the host defence mechanisms. However, in the rhizosphere (the portion of soil influenced by the roots), other processes mediated by non-pathogenic microorganisms are of central importance for plant health and nutrition. Numerous studies have demonstrated the capacity of rhizosphere-inhabiting bacteria to suppress root diseases (O'Sullivan and O'Gara, 1992). Several basic mechanisms of the bacterial-induced biocontrol of plant pathogenic fungi have been described (Compant *et al*., 2005), particularly concerning the *Pseudomonas* genus (Haas and Défago, 2005): antibiosis, fungistasis, competition for nutriments, modification of the biophysical root environment, active exclusion of pathogenic fungi from the rhizosphere, detoxification of pathogen virulence factors and the induction of plant disease resistance.

Because of the complexity of the multitrophic interactions between roots, fungal pathogens and rhizobacteria, most investigations on the mechanisms of soil-borne disease development have focused primarily on only two of the partners in these tripartite interactions (Compant *et al*., 2005; Minerdi *et al*., 2008). Thus, antibiosis and fungistasis have been explained mainly by the production of antibiotic metabolites, antifungal compounds and lytic enzymes by rhizobacteria. Despite further studies on the complex regulatory gene network controlling the production of such effectors by rhizobacteria, little information is available concerning their effects on fungal pathogenicity and virulence. For example, the *Pseudomonas fluorescens* KD strain reduces the activity level of the pectinase polygalacturonase (a key pathogenicity factor) from *Pythium ultimum* on cucumber (Rezzonico *et al*., 2005). In contrast, *P. fluorescens* strains induce

laccase activity, enzymes putatively involved in the pathogenicity of *Rhizoctonia solani* (Crowe and Olsson, 2001).

Fine-tuned communication occurs between fungi and bacteria that can explain the antagonistic or mutualistic associations (Tarkka *et al*., 2009). At a transcriptional level, the fungus influences physiological traits of rhizobacteria, as shown during the time course confrontation between the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) and a strain of *P. fluorescens* (Barret *et al*., 2009a). Similarly, fungal genes of plant symbiotic fungi are differentially expressed during confrontations with bacteria (Deveau *et al*., 2007; Schrey *et al*., 2005): the responsive fungal genes include those involved in signalling pathways, metabolism, cell structure, cell growth response, recognition processes, transcriptional regulation or primary metabolism.

Biocontrol bacteria can also act indirectly through the plant by inducing host defence responses that limit the invasion of the root by a fungal pathogen and change the fungal pathogenicity process. Induced systemic resistance triggered by rhizobacteria has been demonstrated in Arabidopsis (Van Wees *et al*., 1997) and in other plants such as wheat and rice (De Vleesschauwer *et al*., 2008; Shoresh *et al*., 2010). This phenomenon operates through the accumulation of defence compounds and the activation of plant enzymes involved in defence reactions (Van Loon *et al*., 1998). For example, *Bacillus pumilus* 7 km and *P. fluorescens* CHAO have been shown to enhance the defence response of wheat roots inoculated with *Ggt* by increasing the activities of wheat peroxidases (which catalyse the formation of lignin) and glucanases (which cause the lysis of fungal cell walls) (Sari *et al*., 2007, 2008).

Therefore, in addition to the well-documented biocontrol strategies of rhizobacteria, there is a lack of knowledge about the way in which these bacteria interfere with fungal pathogenicity *in planta*. To highlight such an interaction, we propose herein to investigate the tripartite interaction between wheat roots, the fungus *Ggt* and the *P. fluorescens* Pf29Arp strain.

The filamentous fungus *Ggt* causes take-all disease, considered to be one of the most serious diseases of wheat worldwide. The pathogen infects healthy wheat roots via infectious hyphae that develop from mycelium surviving saprophytically in the dead root debris of a previous wheat crop. The disease begins by penetrating the cortical cells of the root and progresses upwards into the base of the stem, consequently disrupting the flow of water and causing the premature death of the infected plant. The *P. fluorescens* Pf29Arp strain provides biological control of take-all of wheat and decreased disease severity when inoculated into the soil (Chapon *et al*., 2002). Pf29Arp is a root-colonizing bacterium isolated from a disease-suppressive soil (Chapon *et al*., 2003). The mechanisms by which this antagonistic strain proceeds remain partly unclear, but three methods of biocontrol have been clearly demonstrated: acidification, change in microbial community composition and induced systemic plant resistance (Sarniguet *et al*., 2006).

In fungi and, more particularly, in *Ggt*, some genes have been identified as being potentially involved in their pathogenicity. Among them, some have been shown to code for proteins of the signalling pathway, such as the gene coding for a mitogen-activated protein kinase (MAP kinase: *Gmk1*). This gene is involved in recognition processes and plays a central role in the transduction of extracellular signals in a variety of fungi, and therefore in the regulation of pathogenesis (Kramer *et al*., 2009; Zhao *et al*., 2007). An association between the degree of virulence of *Ggt* isolates to wheat and the extracellular production of lytic enzymes and their genes *in vitro*, such as laccases (*Lac*), endo-β-1,4-xylanase (*Xyl*) and β-1,3-exoglucanase (*Exo*) (Pearson, 1974), has been suggested. The gentisate 1,2-dioxygenase-like (*Gdo*) gene could be involved in lignin degradation. At the plant level, few genes have been described as being specific to the plant response towards *Ggt*. Guilleroux and Osbourn (2004) described such genes during a large-scale analysis of gene expression on infection of wheat roots by *Gaeumannomyces graminis* var. *avenae* coding for defence-related genes: glutathione-*S*-transferase (*Gst*), enolase (*Eno*) and cinnamyl alcohol dehydrogenase (*Cin*).

The influence of biotic stresses on *Ggt* pathogenesis-related gene expression *in planta* has not been reported. Consequently, in this article, we explore the effects of antagonistic bacteria on such genes in *Ggt*. In order to do so, an *in vitro* system allowing interactions between *Ggt* and wheat roots in the absence or presence of the antagonistic bacterium *P. fluorescens* Pf29Arp was used. In parallel with the assessment of disease reduction by Pf29Arp, the root colonization rates by bacteria and fungus and the transcript levels of candidate fungal pathogenicity and plant-induced genes were monitored during the infection process. Bacterial inoculation changed fungal pathogenesis and probably enhanced basal plant defence.

RESULTS

Biocontrol of take-all by Pf29Arp

The influence of Pf29Arp on *Ggt* pathogenesis was examined 4, 7 and 10 days after inoculation with the fungus in an *in vitro* bioassay. The incidence of attacked roots (Fig. 1a) and the severity of disease (Fig. 1b) were assessed. In uninoculated roots, no necroses were observed. At days 4 and 7, 50.0% and 80.1% of *Ggt*-inoculated roots had developed necrosis, whereas the incidence was significantly lower (29.9% and 58.3%, respectively) with Pf29Arp treatment. At these time points, disease severity was not reduced by Pf29Arp treatment. At day 10, Pf29Arp treatment had no significant effect on disease incidence (88.2%) compared with roots inoculated with *Ggt* alone (94.8%), but

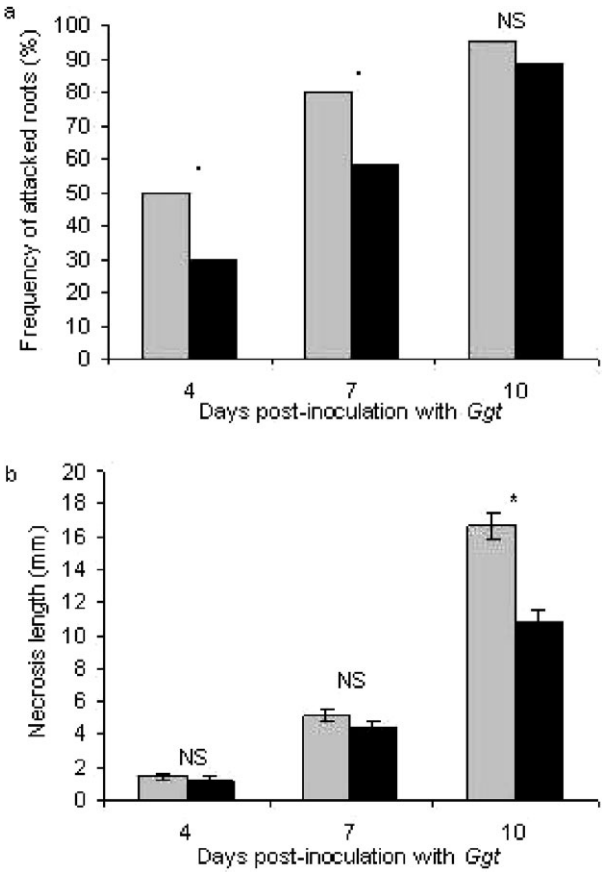

Fig. 1 Effect of *Pseudomonas fluorescens* Pf29Arp on take-all incidence and severity. Wheat seedlings were grown in water agar. Plugs cut from the margin of a *Gaeumannomyces graminis* var. *tritici* (*Ggt*) IV-26/00 culture were inoculated onto roots and incubated for 2 days. At day 2, *Ggt* plugs were removed and 10^6 cells of Pf29Arp were inoculated onto half of the roots. The frequency (%) of necrotic roots (a) and the length (mm) of necrosis (b) were calculated from 136 *Ggt*-inoculated roots (grey histograms) and 144 *Ggt* and Pf29Arp-treated roots (black histograms) involving three biological repetitions. The means of disease severity were performed using only nonzero values (i.e. only for roots with lesions). Error bars represent standard errors of the means. Differences between treatments were analysed by analysis of variance (*$P < 0.05$; •$P < 0.10$; NS, not significant).

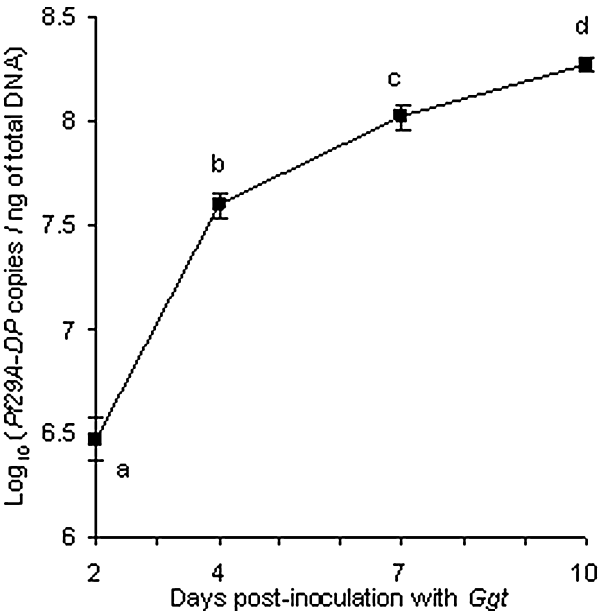

Fig. 2 Colonization of *Pseudomonas fluorescens* Pf29Arp on roots. The quantification of Pf29Arp bacteria was achieved by the quantitative polymerase chain reaction (qPCR) amplification of the *Pf29A-DP* sequence in the *in vitro* confrontation assay from *Gaeumannomyces graminis* var. *tritici* (*Ggt*) and Pf29Arp-inoculated roots. The calculation of the quantity of Pf29Arp DNA was based on a standard curve established with serial dilutions of a known Pf29Arp DNA quantity. Results are expressed as $\log_{10}$(number of amplified fragment copies/ng of total DNA). Results are expressed as the means of three biological replicates each containing three technical replicates. Error bars represent standard errors of the means. Means with different letters are statistically significantly different according to the analysis of variance test ($P < 0.05$).

disease severity was reduced significantly (lesions of 10.8 ± 0.7 mm in length with Pf29Arp treatment vs. lesions of 16.6 ± 0.8 mm in length in the absence of bacteria; corresponding to a 35% reduction in disease severity).

Quantification of bacterial Pf29Arp populations

Bacterial colonization was monitored by amplification of a Pf29Arp-specific sequence (*Pf29A-DP*). The primer set for this assay gave a unique band of the expected size with bacterial DNA and did not amplify any target in the fungus alone, wheat alone or *Ggt*-infected wheat.

The population density of Pf29Arp (Fig. 2) was monitored from day 2 (20 min after bacterial inoculation). A significant increase in the density of Pf29Arp was detected, with $\log_{10}$ bacterial multiplication rates of 1.2 between days 2 and 4, 1.1 between days 4 and 7, and 1.0 between days 7 and 10.

Assay of *Ggt* root colonization

To quantify the amount of pathogen DNA, a portion of the fungus *18S rDNA* was specifically amplified. The set of primers selected provided the most consistent and *Ggt*-specific DNA amplification of a single amplicon. No amplification was observed in roots that were not infected by *Ggt*.

The amount of *Ggt* DNA increased significantly over the experimental period in both conditions, with or without Pf29Arp (Fig. 3). When bacteria were inoculated, the growth rate of *Ggt* between days 4 and 7 was only marginally slower relative to treatment without bacterial inoculation (2×10^8 and 5×10^8, respectively). In contrast, between days 7 and 10, the fungal colonization rate was slightly higher in the presence than in the

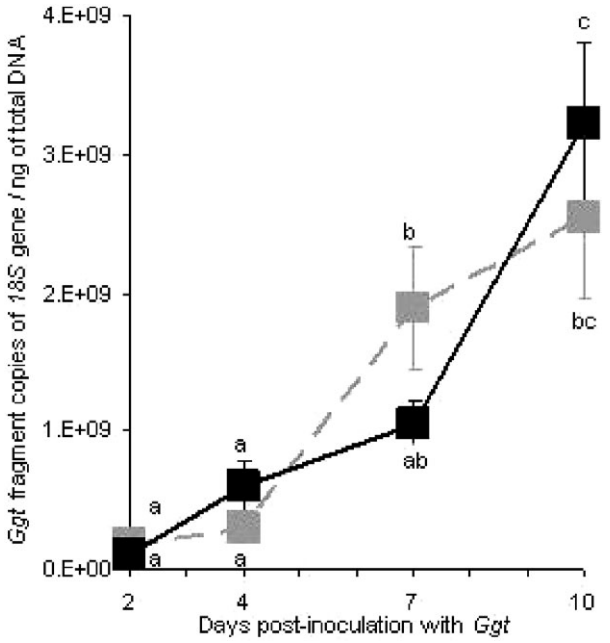

Fig. 3 Root colonization of *Gaeumannomyces graminis* var. *tritici* (*Ggt*). The quantification of *Ggt* 18S DNA was achieved by the quantitative polymerase chain reaction (qPCR) amplification of a region of the 18S sequence in the *in vitro* confrontation assay from *Ggt*-inoculated roots (grey squares) and from *Ggt* and Pf29Arp-inoculated roots (black squares). The calculation of the quantity of *Ggt* DNA was based on a standard curve established with serial dilutions of a known *Ggt* DNA quantity. Results are expressed as the means of three biological replicates each containing three technical replicates. Error bars represent standard errors of the means. Means with different letters are statistically significantly different according to the analysis of variance test ($P < 0.05$).

absence of Pf29Arp (7×10^8 and 2×10^8, respectively). However, for each time period, no significant difference in the abundance of *Ggt* DNA was observed in either the presence or absence of bacteria.

Development and validation of the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) method

An accurate qRT-PCR method was developed and validated to quantify fungal genes coding for 18S rRNA (*18S*), the three laccases described in *Ggt* (*Lac1*, *Lac2*, *Lac3*), endo-β-1,4-xylanase (*Xyl*), β-1,3-exoglucanase (*Exo*), a MAP kinase (*Gmk1*) and gentisate 1,2-dioxygenase-like (*Gdo*). The wheat genes under study code for glutathione-*S*-transferase (*Gst*), enolase (*Eno*) and cinnamyl alcohol dehydrogenase (*Cin*).

The analysis of total RNA using an Agilent 2100 bioanalyser (Agilent Tech. Inc., La Jolla, CA, USA) (Fig. 4) revealed that the *Ggt 18S rRNA* is larger than that of wheat. The fungal *18S rRNA* band was faint in inoculated samples beginning at day 4, and was stronger at days 7 and 10. Similarly, the 23S band of Pf29Arp was detectable starting from day 7. The general profiles of RNA showed no degradation of the samples.

For qRT-PCR gene expression studies, endogenous housekeeping genes are commonly used as references for quantitative analysis. However, in our system, many commonly used housekeeping genes (tubulin, actin and polymerase G) varied with the experimental conditions (data not shown) and were not valid internal controls. To avoid the variation of normalizers for qRT-PCR, regardless of the status of the fungus, a set of two external RNA quality controls for RNA expression analysis was used. The two external controls, *Wn1* and *Wn2* RNA, were obtained by the *in vitro* transcription of two specific PCR products from a pea aphid expressed sequence tag (EST) clone containing the *Wunen* gene (see Experimental procedures). The specificity and efficacy of the two primer sets were analysed by qRT-PCR performed with RNA from *Ggt*, Pf29Arp and wheat, and with *Wn1* and *Wn2* RNA. The *Wn1* and *Wn2* designed primers did not amplify fungal, bacterial and wheat RNA, but efficiently amplified aphid samples as a single product of the expected size (data not shown). Then, amplifications were also carried out using serially diluted aphid cDNA as a template to determine efficiencies. For each primer pair, good PCR efficiencies (87%) and correlation ($r^2 > 0.997$) were observed between the concentration of target cDNA and the observed C_T values (Table 1). To determine the effect of impurities in root extracts on the *Wn1* and *Wn2* qRT-PCR efficiency and reliability, amplifications were conducted with serial dilutions of a mix of inoculated root RNA (containing wheat, fungus and bacteria) and external control RNA. In these experiments, the same correlation was found between C_T and the corresponding cDNA dilution ($r^2 > 0.995$), and the amplification efficiencies were even better (97% and 92% for *Wn1* and *Wn2*, respectively) (Table 1). The quantitative detection levels of the *Wn1* and *Wn2* controls were independent of the host RNA background.

The specificity of qRT-PCR products was checked by amplifications with nucleic acids from *Ggt*, Pf29Arp or wheat as samples, followed by a melting curve analysis, gel electrophoresis and direct sequencing of the amplification products. Each primer pair amplified a single target sequence of the expected size. Each amplicon was specific for the fungal or plant genes studied without cross-reaction with other organisms. For each gene, the correlation between the C_T value and the target cDNA concentration was high ($r^2 > 0.855$), and the efficiencies were good (>86%), irrespective of the presence of nontarget cDNA (Table 1).

Temporal transcript profiling of fungal genes

Real-time qRT-PCR was used to quantify the expression of fungal transcripts at four time points post-inoculation of wheat roots,

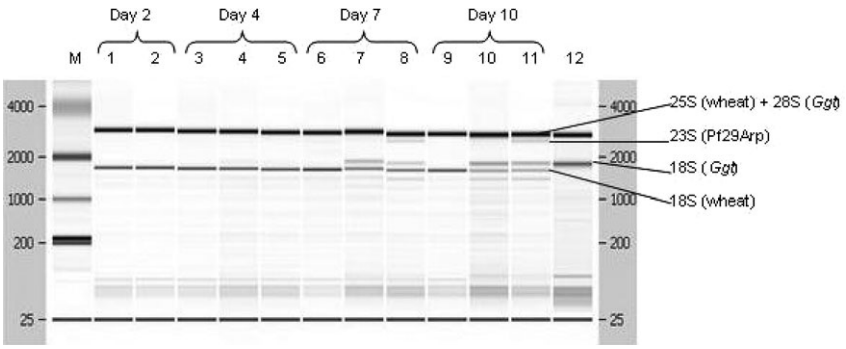

Fig. 4 Quality of root-extracted RNA. The analysis of the total RNA extracted from inoculated roots was performed using an Agilent Bioanalyser 2100. One microlitre of RQ1 DNase-treated total RNA was analysed for each sample. The days post-inoculation with *Gaeumannomyces graminis* var. *tritici* (*Ggt*) are indicated. M, RNA size marker; lanes 1, 3, 6, 9, RNA from uninfected wheat roots; lanes 2, 4, 7, 10, RNA from wheat roots infected with *Ggt*; lanes 5, 8, 11, RNA from wheat roots infected with *Ggt* and Pf29Arp; lane 12, RNA from soil-borne pathogenic fungus IV-26/00.

Table 1 Efficiency of quantitative polymerase chain reactions (qPCRs).

		Standard curve established with cDNA (from aphid*, bacteria†, mycelium‡ or wheat§ RNA), or with DNA from fungus¶, as template			Standard curve established with cDNA from a mix of *Wn1* RNA, *Wn2* RNA and inoculated root RNA as template		
Gene target	Organism	Slope	*E* (%)	r^2	Slope	*E* (%)	r^2
Wn1	*Acyrthosiphon pisum*	–3.68*	87	0.998	–3.40	97	0.977
Wn2	*Acyrthosiphon pisum*	–3.68*	87	0.997	–3.54	92	0.995
Pf29A-DP	*Pseudomonas fluorescens*	–3.39†	97	0.846	/	/	/
18S rDNA	*Gaeumannomyces graminis* var. *tritici*	–3.65¶	88	0.996	/	/	/
18S rRNA	*Gaeumannomyces graminis* var. *tritici*	–3.51¶	93	0.961	–3.33	99	0.995
Lac1	*Gaeumannomyces graminis* var. *tritici*	–3.97‡	79	0.967	–3.61	89	0.953
Lac2	*Gaeumannomyces graminis* var. *tritici*	–3.48‡	94	0.995	–3.45	95	0.896
Lac3	*Gaeumannomyces graminis* var. *tritici*	–3.72‡	86	0.955	–3.47	94	0.930
Gmk1	*Gaeumannomyces graminis* var. *tritici*	–3.74‡	86	0.980	–3.71	86	0.975
Xyl	*Gaeumannomyces graminis* var. *tritici*	–3.60‡	90	0.857	–3.37	98	0.952
Exo	*Gaeumannomyces graminis* var. *tritici*	–3.52‡	92	0.850	–3.31	100	0.968
Gdo	*Gaeumannomyces graminis* var. *tritici*	–3.28‡	100	0.985	–3.57	91	0.942
Gst	Wheat (*Triticum aestivum*)	–3.40§	97	0.867	–3.62	89	0.901
Eno	Wheat (*Triticum aestivum*)	–3.40§	97	0.907	–3.60	90	0.976
Cin	Wheat (*Triticum aestivum*)	–3.26§	100	0.860	–3.47	94	0.855

Wn1, 3′ *Wunen* expressed sequence tag (EST) region amplified in quantitative reverse transcription-polymerase chain reaction (qRT-PCR); *Wn2*, 5′ *Wunen* EST region amplified in qRT-PCR; *Pf29A-DP, Pseudomonas fluorescens* strain Pf29Arp detection probe; 18S rDNA, PCR fragment consisting of the 3′ 70 nucleotides of the 18S gene and a part of the ITS1 DNA sequence; *18S rRNA*, PCR fragment corresponding to a part of the 18S gene; *Lac1*, laccase 1; *Lac2*, laccase 2; *Lac3*, laccase 3; *Gmk1*, mitogen-activated protein (MAP) kinase; *Xyl*, endo-β-1,4-xylanase; *Exo*, β-1,3-exoglucanase; *Gdo*, gentisate 1–2 dioxygenase-like; *Gst*, glutathione-*S*-transferase; *Eno*, enolase; *Cin*, cinnamyl alcohol dehydrogenase.

From the slope of each standard curve, the PCR amplification efficiency *E* was calculated according to the equation $E = 10^{-1/\text{slope}} - 1$ (Rasmussen, 2001). The correlations (r^2) between the concentration of target DNA or cDNA and the observed C_T values were calculated.

with or without Pf29Arp bacterial co-inoculation (Fig. 5). At each time point, the sample was a mix of fragments from colonized roots that may or may not be necrotic. As expected, the real-time qRT-PCR amplification of root extracts yielded no amplification for control treatments (uninoculated plants). In the inoculated tissue samples, the amounts of each fungal gene transcript were first corrected with the *Wn1* and *Wn2* normalization factor. The *Ggt* transcript levels were then expressed relative to the fungal DNA amount quantified by *18S rDNA* qPCR corrected by DNA concentration. This enabled the *Ggt* colonization rates during the time course to be accounted for.

Fungal *18S rRNA* appeared to be affected significantly by the treatment in our study (Fig. 5), thus making it unsuitable for use as an internal control in relative qRT-PCR.

Without Pf29Arp co-inoculation, most of the genes showed transcript levels that were stable between days 2 and 4 after inoculation with *Ggt* (*18S rRNA, Lac1, Lac2, Exo, Gmk1* and *Gdo*). In contrast, *Lac3* gene expression decreased significantly

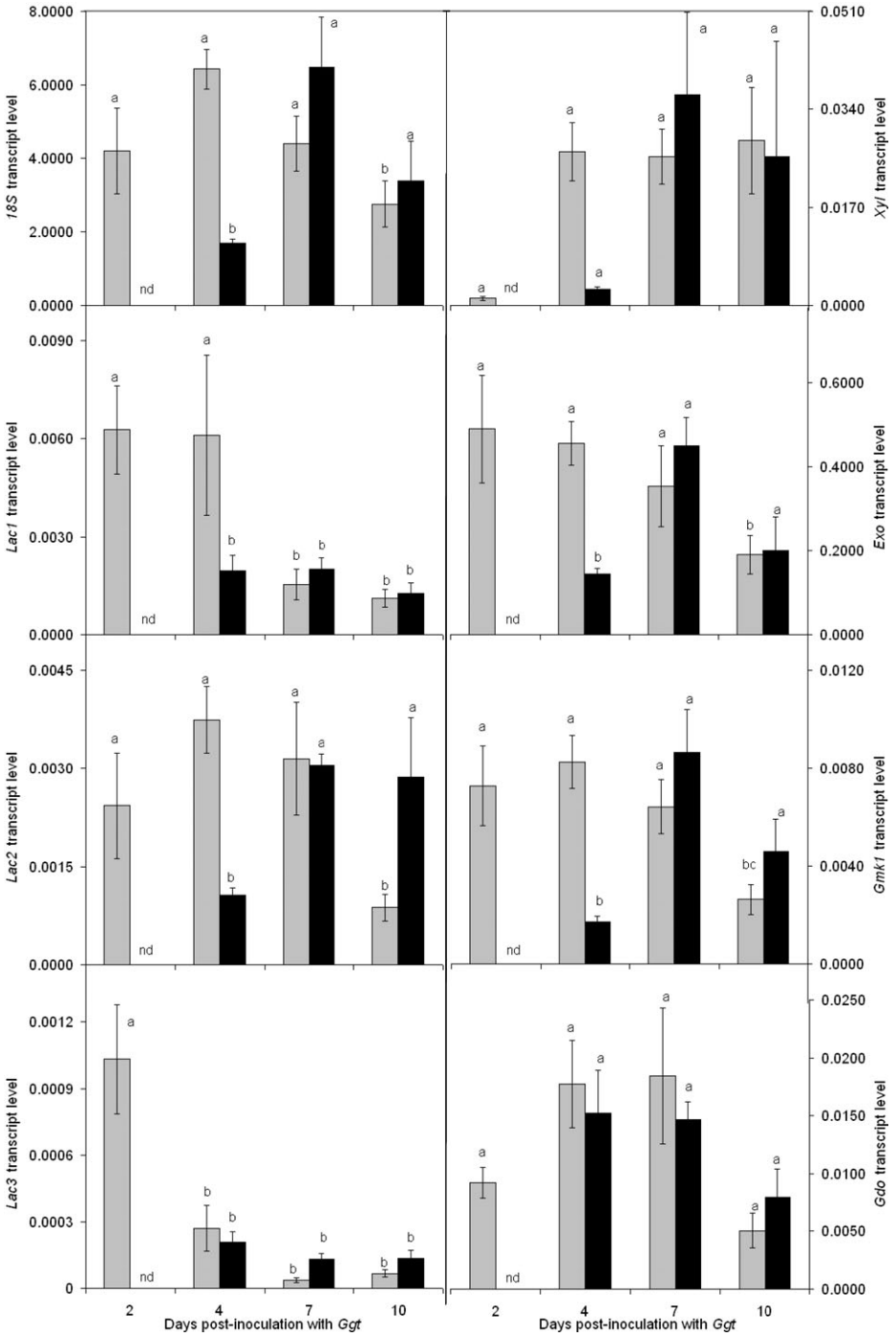

Fig. 5 Transcript profiles of fungal genes at different stages of interaction with roots and Pf29Arp bacteria. *Gaeumannomyces graminis* var. *tritici* (*Ggt*) gene expression profiles were obtained after quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on RNA extracted from *Ggt*-inoculated roots at days 2, 4, 7 and 10 (grey histograms), and from *Ggt* and Pf29Arp-inoculated roots at days 4, 7 and 10 (black histograms). The studied genes were as follows: *18S rRNA*, PCR fragment corresponding to a part of the 18S gene; *Lac1*, laccase 1; *Lac2*, laccase 2; *Lac3*, laccase 3; *Xyl*, endo-β-1,4-xylanase; *Exo*, β-1,3-exoglucanase; *Gmk1*, mitogen-activated protein (MAP) kinase; *Gdo*, gentisate 1–2 dioxygenase-like. The expression of each gene of interest was calculated by dividing the quantities for each sample by the geNorm normalization factor from the external RNA controls (*Wn1* and *Wn2*). The value was then divided by the ratio *Ggt* 18S DNA/total DNA. Each value is the mean of three biological replicates and three technical replicates. Error bars represent standard errors of the means. Means with different letters are statistically significantly different according to the analysis of variance test ($P < 0.05$). nd, nonmeasured value.

between days 2 and 4, and the *Xyl* transcript level was barely detectable at day 2 and increased at day 4, although the difference was not statistically significant at $P = 0.05$, probably because of missing values in *Xyl* qRT-PCR data. From 4 days post-inoculation with *Ggt*, the expression of most of the genes decreased gradually each day for *18S rRNA*, *Lac2*, *Exo* and *Gmk1* transcripts and dramatically at day 7 for the *Lac1* transcript. The *Lac3*, *Xyl* and *Gdo* transcripts displayed a stable and high expression until day 10.

In roots co-inoculated with *Ggt* and Pf29Arp, expression of the *18S rRNA*, *Lac1*, *Lac2*, *Exo* and *Gmk1* genes was reduced significantly at day 4, relative to *Ggt*-infected roots, but this was not observed for the *Lac3*, *Xyl* and *Gdo* transcripts (Fig. 5). Seven days after inoculation with the fungus, no further differences in the levels of gene transcripts were observed between the treatments with or without Pf29Arp. At day 10, the genes were not differentially expressed in the presence or absence of Pf29Arp, except for a higher, but not significant, expression ($P = 0.08$) of the *Lac2* gene in the combined Pf29Arp and *Ggt* treatment relative to that in the *Ggt* condition alone.

Compared with *18S rRNA* gene expression, depicted as a marker of the whole transcription level, Pf29Arp treatment led to two expression profiles: *Lac1*, *Lac2*, *Exo* and *Gmk1* genes displayed an expression pattern similar to that of *18S rRNA* at each time point, and *Lac3*, *Xyl* and *Gdo* genes showed completely different expression profiles.

Finally, as the amplification efficiencies of all *Ggt* genes of interest and controls were shown to be equivalent, and the same baseline cycles were set for all quantifications, this allowed for a comparison between the global expression levels and the different genes under study. Not surprisingly, the highest abundance was found for *18S rRNA* expression. Within the laccase family, *Lac1* was the most expressed and *Lac3* was the least expressed (about 10 times lower).

Temporal transcript profiling of wheat genes (Fig. 6)

The *Gst* gene exhibited a similar expression during all stages of the time course in the uninfected and healthy control roots. In *Ggt*-infected roots, the *Gst* gene showed high fluctuations, with

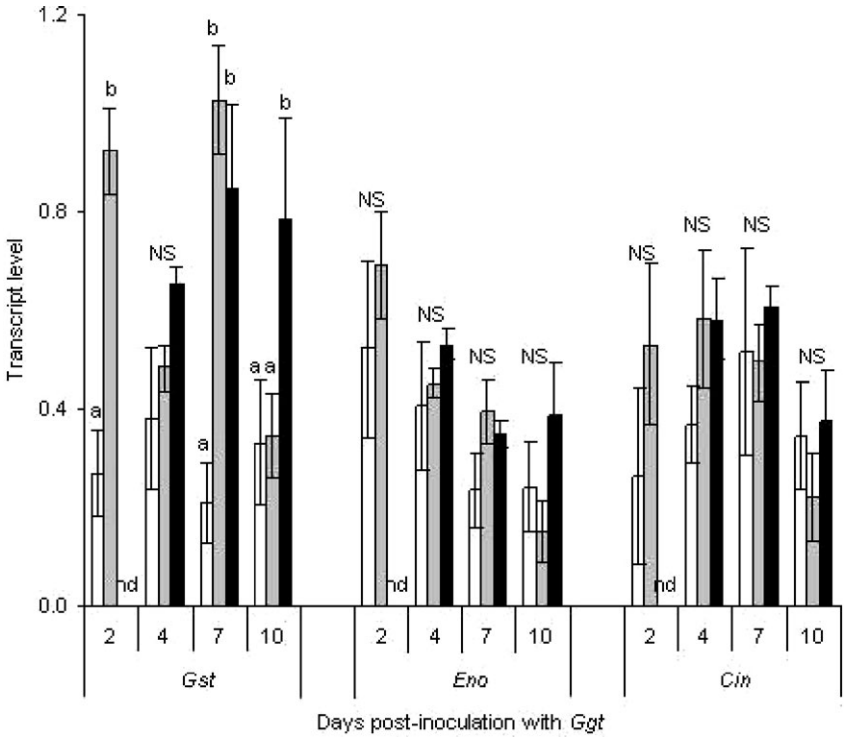

Fig. 6 Transcript profiles of wheat genes at different stages of the interaction with *Gaeumannomyces graminis* var. *tritici* (*Ggt*) and Pf29Arp bacteria. Root gene expression profiles were obtained after quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on RNA extracted from healthy noninoculated roots at days 2, 4, 7 and 10 (white histograms), from *Ggt*-inoculated roots at days 2, 4, 7 and 10 (grey histograms), and from *Ggt* and Pf29Arp-inoculated roots at days 4, 7 and 10 (black histograms). The studied genes were as follows: *Gst*, glutathione-*S*-transferase; *Eno*, enolase; *Cin*, cinnamyl alcohol dehydrogenase. The expression of each gene of interest was calculated by dividing the quantities for each sample by the geNorm normalization factor from external RNA controls (*Wn1* and *Wn2*). The value was then divided by the wheat DNA/total DNA ratio. Each value is the mean of three biological replicates and three technical replicates. Error bars represent standard errors of the means. At each time, means of the same gene followed by the same letter are not significantly different according to the analysis of variance test ($P < 0.05$). nd, nonmeasured value; NS, not significant.

significant down-regulation between days 2 and 4 and between days 7 and 10, and a significant increase between days 4 and 7. At days 2 and 7, *Ggt* treatment led to a strong up-regulation of *Gst* expression compared with control roots. Co-inoculation with Pf29Arp resulted in a high, stable induction of *Gst* and an up-regulation compared with control roots at day 7 and compared with control and *Ggt*-infected roots at day 10.

Eno expression was stable for each treatment during the time course, except for a significant diminution between days 2 and 10 in *Ggt*-infected roots. At no time during the time course was *Eno* expression affected by any of the treatments.

Cin gene expression rates were not affected significantly, either over time or by treatment.

DISCUSSION

In this study, we examined the alteration of certain fungal transcripts related to pathogenesis, as well as the alteration of certain host response genes, during interaction of the fungus with antagonistic bacteria inoculated at the surface of wheat roots.

Following from Barret *et al*. (2009b), a time course experimental design in gnobiotic conditions was established to observe tripartite interactions and to obtain multiple dynamic parameters: root infection progress, bacterial and fungal root colonization kinetics, and fungal and plant gene expression kinetics. For this, methods were adapted to obtain high-quality total DNA and RNA extracts, high specificity for PCR gene amplification in a complex matrix and high significance in gene expression assessment with the help of an innovative external reference method.

In these experimental conditions, the inoculation with Pf29Arp 2 days after the inoculation of the root with *Ggt* led to a decrease in the incidence of take-all disease at 4 and 7 days of interaction. The disease severity estimated from the root necrosis length was not affected by bacterial treatment at days 4 and 7, but decreased by 35% at day 10, when compared with disease severity without bacteria. Thus, Pf29Arp exhibited biocontrol activity, first by affecting the efficiency of root infection and then by affecting necrosis extension.

The density of detectable bacterial populations increased from day 2 to day 10. The efficient bacterial colonization of the environment is a crucial factor with regard to the efficacy of fluorescent pseudomonads as suppressors of soil-borne diseases (Barret *et al*., 2009b; Chapon *et al*., 2003). Hence, this tripartite experimental design is well suited as an *in vitro* model for the study of antagonistic interactions.

At the same time points, the amounts of *Ggt* mycelium colonizing the roots were assessed by qPCR of part of the *18S rDNA* gene. Different parameters have been used to estimate the biomass of a pathogenic fungus growing within its host: the activity of reporter genes, such as β-glucuronidase (Olivier *et al*., 1993), the accumulation of constitutively expressed fungal mRNA (Avrova *et al*., 2003), the quantity of fungal DNA vs. host DNA with multiplex qPCR (Karlsson *et al*., 2007) or qPCR related to the gene copy number (Berruyer *et al*., 2006). In our system, no genes showed a stable transcription rate under different experimental conditions, excluding specific internal mRNA quantification as an indicator of fungal biomass. Moreover, the simultaneous DNA extraction of three organisms rendered difficult the use of a multiplex qPCR. To overcome these difficulties, the *18S rDNA* gene target was chosen because it is *Ggt* species specific, does not depend on the transcription activity and links the quantity of DNA to the fungal biomass. One limit is that DNA techniques do not discriminate between dead and living mycelium. One hypothesis is that, in living root tissues, free DNA from dead cells is quickly destroyed. The augmentation of *Ggt* DNA quantity throughout the time series indicates that mitoses occurred in active mycelium. The method also measured total mycelium DNA outside and inside the roots.

Bacterial inoculation had no significant effect on the amounts of fungal mycelium at any point of the time course experiment, whereas it altered the development of take-all. The antagonistic effect is usually explained by multiple mechanisms, including the toxicity of different bacterial metabolites (antibiotics, hydrolytic enzymes, siderophores, volatile metabolite) leading to antibiosis (fungal death) or fungistasis (growth slowing or stopping) (de Boer *et al*., 2003; Raaijmakers *et al*., 2009). In our study, a slightly smaller increase between days 4 and 7 in the presence of Pf29Arp resulted in a shorter necrosis length seen at this late stage of infection (day 10), indicating that fungal growth precedes visible lesion formation. However, biocontrol using Pf29Arp did not affect the temporal fungal population density, suggesting that it may operate through a mechanism different from antibiosis, fungistasis or competition for space. The interaction of *Ggt* with Pf29Arp outside the plant may result in a reduction in the rate of entry of *Ggt* cells into the plant, as suggested during the interaction between *Ggt* and the biocontrol strain CHAO (Sari *et al*., 2008).

The expression of pathogenesis-related and plant response genes was monitored by qRT-PCR, a method that requires reference genes to compare the expression of all genes. However, in our system, the commonly used housekeeping genes could not be employed as internal controls. This drawback has been described in many studies of gene quantification (Kim *et al*., 2003). To overcome the variation of normalizers for qRT-PCR regardless of the status of the fungus, we determined a set of two external RNA quality controls for RNA expression analysis according to Liu and Slininger (2007). We developed quality controls for qRT-PCR analyses using external nucleic acids (from aphid) and successfully applied them in fungal and wheat gene expression. These new quality controls ensured the reliability and

reproducibility of gene expression data, and provided unbiased normalization references for validation, quantification and the estimation of gene expression experiments because they satisfied the following features: (i) they were reverse transcribed from a known quantity of target RNA that was similar from sample to sample, thus allowing the normalization of RT efficiency; (ii) their expression level was chosen to be close to that of the target genes; and (iii) the primers for their amplification were an aphid-specific species preventing nonspecific amplification with fungal or wheat RNA. In this study, despite the low total RNA extracted from infected plant tissue and the low proportion of fungal RNA relative to plant RNA, an adequate method of qRT-PCR, with synthetic external RNAs as controls for qRT-PCR analyses, was used to accurately quantify the expression of candidate genes.

Genes were chosen from those described during the interactions of pathogenic fungi with their hosts, which eventually play a role in root pathogenesis and adaptation. In most total gene cataloguing studies of interactions between a fungus and its host, few fungal ESTs have been recovered, for example, from *Fusarium graminearum*-infected wheat leaves (Kruger *et al*., 2002), *Magnaporthe oryzae*-infected rice leaves (Jantasuriyarat *et al*., 2005) or *Ggt*-infected wheat roots (Guilleroux and Osbourn, 2004). In this study, all the selected fungal genes were shown to be expressed on roots and exhibited altered levels of expression during the time course of interaction with wheat and Pf29Arp. The observed differences in expression reflect the mean disease state because the sampled root fragments included necrotic and symptomless roots. The *18S rRNA, Lac1, Lac2, Exo* and *Gmk1* genes displayed a significant decrease in expression level in roots co-inoculated with Pf29Arp, 4 days after *Ggt* infection, but this was not found for *Lac3, Xyl* and *Gdo* genes. The *18S rRNA* gene that could be considered as an indicator of transcription activity (and therefore metabolic activity) showed decreased expression in *Ggt* in contact with Pf29Arp. At this point in the time course, the frequency of attacked roots showed a significant decrease. Infection by *Ggt* requires the penetration of epidermal cells along the root. Furthermore, host colonization and survival of the pathogen may depend on the pathogen's ability to degrade cell wall constituents. Laccases are involved in the degradation of lignin for the penetration of plant cell walls during the infection process, melanin synthesis and the oxidation of humic acids and manganese ions. Three genes encoding laccase enzymes have been cloned from *Ggt* (Litvintseva and Henson, 2002). In our study, the three laccases displayed different expression profiles. Litvintseva and Henson (2002) reported that *Lac1* was transcribed constitutively, *Lac2* was copper inducible and *Lac3* was found only *in planta*. Pf29Arp led to a decrease in *Lac1* and *Lac2* levels at day 4, but did not affect the expression of *Lac3*. Generally, a strong induction of laccase is described as a means of resisting antagonists in several higher fungi (Velasquez-Cedeno *et al*., 2004; Zhang *et al*., 2006). This was also demonstrated in *Rhizoctonia solani* challenged with *Pseudomonas* strains producing antifungal compounds (Crowe and Olsson, 2001). In our pathosystem, the decrease in the expression of laccase on co-inoculation with Pf29Arp may reflect a mechanism of antagonism by inhibition of the pathogenesis of the fungus. This concerns particularly the *Lac2* gene encoding a secreted laccase of *Ggt* that has been purified and catalyses the polymerization of a fungal melanin precursor (Edens *et al*., 1999). The functional activity of *Lac3* has never been demonstrated.

Similarly, the lytic enzyme EXO that is secreted by *Ggt* may be involved in the pathogenesis of the take-all fungus through the degradation of glucan and callose in cell wall appositions, such as lignitubers (Dori *et al*., 1995; Yu *et al*., 2009) formed after infection. The decrease in fungal *Exo* gene expression in the presence of Pf29Arp could be a mode of bacterial antagonistic activity.

The *Gmk1* gene was also down-regulated by Pf29Arp 4 days after inoculation with *Ggt*. The MAP kinases work in a cascade of hierarchical components, and their involvement in early communication with the plant has been emphasized in several systems (Lengeler *et al*., 2000; Zhao *et al*., 2007). In the rice blast pathogen *M. grisea*, the MAP kinase (*pmk1*) has been shown to be required for virulence, because *M. grisea pmk1*-deficient mutants are unable to infect rice leaves (Dufresne and Osbourn, 2001). A *pmk1*-related MAP kinase from *G. graminis* has been shown to functionally complement the *M. grisea pmk1* mutants, and is likely to be required for root infection. In an antagonistic strain of *F. oxysporum*, the levels of expression of three genes involved in fungal pathogenesis (MAP kinase, chitin synthase and pectate lyase) were silenced by the interaction with a consortium of ectosymbiotic bacteria (Minerdi *et al*., 2008). As the *Gmk1* gene appeared to be at the crossroads between the expression of pathogenicity and environmental sensing, it may be a plausible target for bacterial influence.

The site of attack of the XYL enzyme is within the polysaccharide backbone. A number of cereal pathogens have been reported to produce XYL activity when grown on cereal cell walls (Southerton *et al*., 1993). In infected wheat roots, the density of xylan in the cell walls was reduced significantly when compared with the corresponding cell walls of healthy wheat roots (Kang *et al*., 2000). The degradation of these cell wall components in infected wheat roots demonstrates indirectly that *Ggt* may secrete corresponding cell wall-degrading enzymes, such as XYL, during infection of wheat roots. *Xyl* gene expression in *Ggt* on infection of wheat roots in our experiment, and in *G. graminis* var. *avenae* (Southerton *et al*., 1993) and *M. grisea* on infection of rice leaves (Wu *et al*., 2006), supports the notion of *in planta* active *Xyl*. However, *Xyl* gene transcription is not affected by Pf29Arp inoculation: numerous *Xyl* genes have been described in several phytopathogenic microorganisms and each *Xyl* gene

exhibits a unique expression pattern influenced by specific conditions, including the stage of infection (Belien *et al*., 2006; Hatsch *et al*., 2006).

The GDO enzyme catalyses the chemical reaction leading to maleylpyruvate with 2,5-dihydroxybenzoate (or gentisate) as substrate (Dodge and Wackett, 2005). Gentisate serves as the key intermediate in the biodegradation of a large number of simple and complex aromatic compounds by microorganisms. GDO has been purified and characterized in many bacteria, but not yet in fungi. The *Gdo* gene has been used as a polymorphic marker to discriminate between the G1 and G2 *Ggt* genotype groups (Daval *et al*., 2010). The *Gdo* gene is expressed in *Ggt* mycelium growing *in vitro* and, herein, we provide the first evidence that it is expressed *in planta*. Its transcription level in *Ggt* was not altered as the infection progressed over time, either with or without bacterial inoculation. However, it is difficult to propose its role in virulence without further study.

As Pf29Arp can act on wheat gene expression, and as the information regarding the genetic factors that determine the outcome of interactions between *Ggt* and plant roots is limited, the expression of three wheat genes during interaction with *Ggt* and with antagonistic bacteria has also been studied. As a result of the lower rate of pathogen cell entry in the presence of Pf29Arp, shown by the decrease in disease incidence, there was sufficient time for the plant to develop resistance mechanisms in order to defend itself against the pathogen.

As demonstrated in the report of Guilleroux and Osbourn (2004), in this study, the *Gst* gene was expressed only weakly in control wheat plants and up-regulated during fungal infection. Treatment of the roots with Pf29Arp activated *Gst* expression at days 7 and 10. GSTs are proteins associated with the oxidative burst (Lamb and Dixon, 1997), and they belong to a heterogeneous group of cell-detoxifying enzymes. Elevated GST activities have been found in plants exposed to a wide range of environmental stress effects and microbial infections, and it is now widely accepted that glutathione participates in plant defence against viral, fungal and bacterial infections (Komives *et al*., 1998). For example, the enhanced resistance of melon and tomato roots against *F. oxysporum* coincides with a significant increase in glutathione levels and an up-regulation of *Gst* gene expression (Bolter *et al*., 1993; Medeiros *et al*., 2010). The GST protein is strongly induced in the roots of rice infested by *Azoarcus* sp. (Miché *et al*., 2006), and *Gst* genes are induced in *Brassica napus* after *Sclerotinia sclerotiorum* inoculation (Zhao *et al*., 2009). In Arabidopsis, a transcriptional analysis has identified elicitor-induced acclimatory responses to stress, such as the recovery of the cell redox balance by GST (Blanco *et al*., 2009). Under conditions of stress or pathogen attack, as in the case of powdery mildew, the *Gst* gene has been found to be highly induced in wheat (Chao *et al*., 2006; Mauch and Dudler, 1993).

In our study, the expression of the *Eno* and *Cin* genes did not differ between treatments or over time (except for a diminution in the *Eno* gene in *Ggt*-inoculated roots between days 2 and 10). ENO is a ubiquitous enzyme that catalyses the dehydration conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway, and CIN is an enzyme participating in phenylpropanoid biosynthesis and is also involved in lignin biosynthesis. In wheat infected by *G. graminis* var. *avenae* (Guilleroux and Osbourn, 2004), the wheat *Eno* gene was up-regulated during fungal infection and the *Cin* gene was an EST from the unsubtracted cDNA library of infected wheat. The differences in the results between the two studies can be explained by the difference in inocula (*Ggt* and *G. graminis* var. *avenae*) and the difference in time course.

These results show that plant defence mechanisms were triggered over time during the infection of roots by *Ggt*, and that some of these mechanisms could be enhanced by Pf29Arp. The evidence provided here shows that such plant responses are local, occurring at the *Ggt* infection site and at the bacterial niche level. At this stage and with this form of experiment, no data could support whether or not this was a systemic response, but it was, without doubt, a local response. The determination of the influence of the bacteria themselves on plant reactions (without disease) was beyond the scope of this study.

This study has demonstrated that the antagonism exerted by rhizobacteria does not act exclusively as antibiosis or fungistasis. When root colonization by *Ggt* mycelium was either unaffected or weakly affected, the expression of plant pathogenesis-related genes was modified by the colonization of roots by rhizobacteria. The changes in the expression profiles of these genes could be related to biocontrol activity. Hence, one gene involved in plant defence responses was also highly induced in the presence of Pf29Arp. The methods and experimental design applied here validated the simultaneous multi-monitoring of the DNA content and expression of different genes in different organisms (plant, fungus and bacterium). With regard to bacterial genes influenced by plant and fungus mycelium (Barret *et al*., 2009b), an exhaustive global transcriptomic approach is now possible, and could lead to the identification of new fungal and plant genes involved in responses to rhizobacteria biocontrol activity.

EXPERIMENTAL PROCEDURES

Plant growth assay and root inoculations

The experimental design for tripartite interactions developed and described by Barret *et al*. (2009b) was applied here. Briefly, 7-day-old seedlings of wheat (*Triticum aestivum* cv. Talent) were laid on water agar in a Petri dish. Roots were accessible for further inoculations by the fungus and the bacterium.

The soil-borne pathogenic fungus *Ggt* IV-26/00 (Willocquet *et al*., 2008) was cultured on potato dextrose agar (Merck, Darmstadt, Germany) at 20 °C for 1 week. The *P. fluorescens* bacterial strain Pf29Arp (Chapon *et al*., 2002) was grown as described previously (Barret *et al*., 2009b).

For the inoculations, an 8-mm^3 plug cut from the edge of the growing *Ggt* colony was placed on each root at a distance of 3 cm below the seed and incubated at 15 °C with a lighting regime of alternating 14 h of light and 10 h of darkness. At day 2, the plugs were removed. For half of the dishes, 10^6 bacterial cells (in 2 × 5-μL aliquots) were deposited on each root right through the deposit of the fungal plug. Seedlings, with or without Pf29Arp, were then incubated at 15 °C with an alternating regime of 14 h of light and 10 h of darkness until days 4, 7 and 10. For each treatment and each time, about 20 different plants were required to provide 60 root fragments.

Controls consisted of healthy roots (without inoculations of either *Ggt* or Pf29Arp). Assays were repeated for three independent experiments.

Root disease assessment

Disease was assessed in the presence and absence of Pf29Arp inoculation at 2, 4, 7 and 10 days after inoculation with *Ggt*. The observations were conducted on 45 roots for each condition (consisting of about 16 seedlings) and independently repeated on three occasions. Disease symptoms were observed using a binocular microscope and quantified with Archimed software (Microvision Instruments, Evry, France). Disease incidence corresponded to the frequency of roots with dark necrotic symptoms and disease severity corresponded to the necrosis length of diseased roots. The mean disease severity was calculated using only nonzero values (i.e. conditional on the presence of a lesion).

Nucleic acid isolation and quality analysis

Sixty root fragments of 7 mm each (obtained from about 20 different plants), located on both sides of the site of the *Ggt* plug deposit, were removed for each independent biological replicate for each treatment. They were ground to a powder with a pestle in liquid nitrogen-chilled mortars with Fontainebleau sand, and separated into two subsets for RNA and DNA isolation.

For DNA extraction, 500-μL aliquots of extraction buffer (120 mM potassium phosphate buffer, pH 8, 0.35 M NaCl, 5% trimethylammonium bromide) were added to the powder. After treatment with proteinase K, a standard phenol–chloroform–isoamyl alcohol (25:24:1) procedure was performed. The DNA yield was determined spectrophotometrically with a Nanodrop (Agilent Tech. Inc.). We make the assumption here that the yield of DNA extracted is the same, sample to sample, for infected plant cells and the fungal or bacterial cells colonizing them, respectively.

Total RNA was extracted from ground tissue in 1 mL of Trizol (Invitrogen, Paisley, Renfrewshire, UK), and contaminating DNA was removed using the RNase-free RQ1 DNase (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. RNA purity and quality were assessed with a Bioanalyser 2100 (Agilent) and quantified with a Nanodrop (Agilent). The efficiency of RNA extraction might differ among plant cells and fungal hyphae, but did not change from sample to sample (Berruyer *et al*., 2006).

Synthesis of external RNA controls

To avoid the variation of normalizers for qRT-PCR, regardless of the status of the fungus, a set of two external RNA quality controls for RNA expression analysis applied to real-time qRT-PCR using SYBR Green was determined. An EST library from the nonhomologous species pea aphid *Acyrthosiphon pisum* was used as a source of candidate sequences. The *Wunen* gene, coding for a phosphatidic acid phosphatase involved in cell signalling, was chosen, and its sequence was examined for similarity against available microbial and wheat genomic sequences using BLAST search. From EST, PCRs were performed in different *Wunen* gene regions to obtain two amplicons that contained the T7 RNA polymerase promoter site. For this purpose, the forward primers were modified with the T7 promoter site (5′-TAATACGACTCACTATAG-3′: Table 2) at the 5′ end, which enabled T7 RNA polymerase-mediated *in vitro* transcription using the PCR products as templates. Each of the DNA oligonucleotides was manually designed using Primer 3. Amplification reactions were performed in a volume of 50 μL containing 1.8 mM $MgCl_2$, 300 μM deoxynucleoside triphosphate (dNTP), 0.6 μM each of forward and reverse primers (Table 2), 1 × reaction buffer, 3 U Ampli Taq DNA polymerase (Roche, Meylan, France) and 50 ng of cDNA. The PCR conditions consisted of an initial denaturation at 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 3 min. These selected amplified sequences, *Wn1T7* and *Wn2T7*, were verified by DNA sequencing (GATC Biotech., Mulhouse, France) and used as templates for *in vitro* transcription with the MEGAscript Kit (Ambion, Austin, TX, USA) to obtain purified *Wn1* and *Wn2* RNA. After treatment by RNase-free RQ1 DNase, RNA purity and quality were checked with a Bioanalyser 2100 (Agilent) and quantified with a Nanodrop (Agilent).

To test the specificity of the primer sets for qRT-PCR, amplifications were performed. *Wn1, Wn2, Ggt*, wheat and Pf29Arp RNA were reverse transcribed as templates. RT was carried out in 20-μL volumes with 500 ng of RNA, 1 μg of oligo-dT primers (random primers for bacterial RNA), 1 × ImPromII reaction buffer, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 20 U of RNasin Ribonuclease Inhibitor and 1 μL of ImProm-II™ (Promega Corp.). The

Table 2 Oligonucleotide primers used in this study.

Gene target*	Source†	Organism	Forward primer	Reverse primer	Amplicon size (bp)	Annealing temperature (°C)
Wn1T7	Sabater-Muñoz *et al*. (2006)	*Acyrthosiphon pisum*	5′-TAATACGACTCACTATAGGGAAGGCCCAACAAATTTACCG-3′	5′-GCGTTTTCGGAGGGATTTAT-3′	336	50
Wn2T7	Sabater-Muñoz *et al*. (2006)	*Acyrthosiphon pisum*	5′-TAATACGACTCACTATAGGGGATCGTCGTACAGCGTTCAT-3′	5′-GGAAATCCAACTGAACCTAAGC-3′	332	50
Wn1	Sabater-Muñoz *et al*. (2006)	*Acyrthosiphon pisum*	5′-TCATTTCCTTCTGGGCATTC-3′	5′-GCACAAAACACTGCCATCAA-3′	146	61
Wn2	Sabater-Muñoz *et al*. (2006)	*Acyrthosiphon pisum*	5′-GTAACGTCGTCCCACTTTCAAT-3′	5′-CTAACGTCGGGCGTAAACTAAT-3′	140	61
Pf29A-DP	AF360119	*Pseudomonas fluorescens*	5′-GCCAACTAGATGTAACAAAAATGACCG-3′	5′-GAGTACCTTCTTACATGGCATGCC-3′	210	61
18S rDNA	FJ771002	*Gaeumannomyces graminis* var. *tritici*	5′-CGAACTCGGTCGTTTAGAGG-3′	5′-GGTATGTTCACAGGGGTTGG-3′	107	60
18S rRNA	FJ771002	*Gaeumannomyces graminis* var. *tritici*	5′-CGTAACAAGGTCTCCGTTGG-3′	5′-AATGATCCCTCCGCTGGT-3′	40	60
Lac1	AJ417685	*Gaeumannomyces graminis* var. *tritici*	5′-CGCTCGAGAGGATCTTTGAC-3′	5′-GTGGATGGGGTGGGTGAG-3′	170	62
Lac2	AJ417686	*Gaeumannomyces graminis* var. *tritici*	5′-TTCCGCTGGTACCTCAACTC-3′	5′-ATGACGTAGGCCCACTTGTC-3′	146	62
Lac3	AJ417687	*Gaeumannomyces graminis* var. *tritici*	5′-ACATGAACTTCCCGCAGAAC-3′	5′-CCGAGCAGGTAGAAGTCGTG-3′	133	60
Gmk1	AF258529	*Gaeumannomyces graminis* var. *tritici*	5′-GAGGGATCTGGTGGACATTG-3′	5′-ATCCAACAGGAAACCTCGTG-3′	160	60
Xyl	AJ249160	*Gaeumannomyces graminis* var. *tritici*	5′-CGACAACTTCGGCGAGTACA-3′	5′-GAGGTACGGATGGCCCAGTA-3′	159	60
Exo	CF554536	*Gaeumannomyces graminis* var. *tritici*	5′-GCGGGACTGGAGTACTACGG-3′	5′-GGGTCCTGCCAGATGGAGAT-3′	179	60
Gdo	FJ717712	*Gaeumannomyces graminis* var. *tritici*	5′-GGGGGCGATTATGTCACTCT-3′	5′-CAAATTTCTCGCTGCCAATG-3′	184	62
Gst	CF554506	Wheat (*Triticum aestivum*)	5′-GAGGAGGACAGAGCCGAGAA-3′	5′-AGCGCCTCAAACCAGAAGAG-3′	161	60
Eno	CF554394	Wheat (*Triticum aestivum*)	5′-TGGGAAAGGGTGTTTCCAAG-3′	5′-CCAGGATTGCATTAGCACCA-3′	177	60
Cin	CF554361	Wheat (*Triticum aestivum*)	5′-GCACACATCAACCCATGGAA-3′	5′-GCTGGAGAAGAACGACGTGA-3′	158	60

*Wn1T7, amplicon of the *Wunen* expressed sequence tag (EST) 3′ region used as *in vitro* transcription template; Wn2T7, amplicon of the *Wunen* EST 5′ region used as *in vitro* transcription template; *Wn1*, 3′ *Wunen* EST region amplified in quantitative reverse transcription-polymerase chain reaction (qRT-PCR); *Wn2*, 5′ *Wunen* EST region amplified in qRT-PCR; *Pf29A-DP, Pseudomonas fluorescens* strain Pf29Arp detection probe; 18S rDNA, PCR fragment consisting of the 3′ 70 nucleotides of the 18S gene and a part of the ITS1 DNA sequence; *18S rRNA*, PCR fragment corresponding to a region of the 18S gene; *Lac1*, laccase 1; *Lac2*, laccase 2; *Lac3*, laccase 3; *Gmk1*, mitogen-activated protein (MAP) kinase; *Xyl*, endo-β-1,4-xylanase; *Exo*, β-1,3-exoglucanase; *Gdo*, gentisate 1–2 dioxygenase-like; *Gst*, glutathione-*S*-transferase; *Eno*, enolase; *Cin*, cinnamyl alcohol dehydrogenase.

†Reference or GenBank accession number.

following parameters were applied: 5 min at 25 °C, 1 h at 42 °C and 15 min at 70 °C. qPCRs were performed as described below. Amplifications of the monitored RNA sequences were achieved using serially diluted cDNA as a template to determine the amplification efficiencies. Melting curve analysis was performed after the qPCR to confirm that the signal, obtained only in the *Wn1* and *Wn2* RNA samples, was the result of a single product of amplification and not caused by primer dimers or an arbitrary amplification.

cDNA synthesis

Total RNA (750 ng) from root fragments was mixed with 100 pg/μL *Wn1* RNA and 0.1 pg/μL *Wn2* RNA as external controls. A reference RNA from pure *Ggt* mycelium was similarly processed. RT was carried out in 30 μL containing 375 ng of random primers, 1 × ImPromII reaction buffer, 3 mM $MgCl_2$, 125 μM of each dNTP, 30 U of RNasin Ribonuclease Inhibitor and 1.5 μL of ImProm-II™ (Promega Corp.). The parameters of RT described above were applied. Reactions without RNA or without reverse transcriptase were performed as negative controls.

Real-time qPCR assays

The oligonucleotides designed using Primer 3 software are described in Table 2.

qPCRs (20 μL) containing 1 μL of cDNA or DNA (diluted 1 : 2 and 1 : 20, respectively), 0.4 μM of each primer and 1 × SybrGreen I Master (Roche) were performed on a LightCycler® 480 Real-Time PCR System (Roche). The qPCR profile consisted of an initial denaturation at 95 °C for 5 min, followed by 50 cycles of 95 °C for 15 s and hybridization–elongation temperature (Table 2) for 40 s. A dissociation analysis was constructed at the end of each run to ascertain whether a single amplicon was generated for each primer used. After qPCR, each type of amplicon was electrophoresed on agarose gels to verify amplification, and was sequenced directly (GATC Biotech.) to confirm that only the target sequence was amplified. No amplification was found in the appropriate controls (water instead of RNA or cDNA, cDNA from uninfected roots, templates from free reverse transcriptase reactions). Real-time qPCR and qRT-PCR data were expressed as means (with standard error of the mean) of three independent biological replicates, each with three technical replicates.

The amounts of bacterial and pathogen DNA in the sample were quantified from day 2 to day 10 in the three biological repetitions of the *in vitro* confrontation assay with a qPCR procedure on a specific *P. fluorescens* strain Pf29Arp detection probe (*Pf29A-DP*: AF360119) and on a portion of the *Ggt* small subunit *18S rDNA* (corresponding to a part of the 18S gene and ITS1 DNA sequences) as targets, respectively. The primers used to amplify the *Pf29A-DP* sequence were designed from a major band specific for Pf29Arp in a random amplification of polymorphic DNA (RAPD) pattern for the specific monitoring of root colonization by Pf29Arp strain (Chapon *et al*., 2002, 2003). Serial 10-fold dilutions of known amounts of purified genomic DNA from Pf29Arp (ranging from 50 to 0.05 ng/μL) and from *Ggt* (ranging from 1700 to 0.17 ng/μL) were used to calculate correlation coefficients and efficiencies (Table 1), and to construct calibration curves. As the quantification procedure showed a linear relationship ($r^2 > 0.846$) between the logarithmic values of bacterial or fungal genomic DNA and real-time qPCR threshold cycles over the range of DNA concentrations examined, the amounts of bacterial and *Ggt* DNA in the samples could be calculated from these standard curves based on their cycle threshold (C_T) values. The threshold level for fluorescence was set at the point at which the fluorescence values of the samples were in the log-linear phase of increase. The cycle at which a sample's signal exceeded the threshold (C_T value) was used to calculate the total DNA amplified. The C_T values determined were plotted against the logarithm of their known initial concentrations, the standard curve generated by linear regression through these points linking observed C_T values with the target concentration. This model was used to calculate the concentration of the Pf29Arp target DNA and *Ggt* DNA in each sample from the observed C_T values. The amounts of bacterial and fungal DNA were expressed as $\log_{10}$(number of amplified fragment copies/ng of total DNA) and as the ratio between the number of amplified fragment copies and the total DNA (ng), respectively.

The *Ggt* candidate genes for transcript profiling corresponded to laccase isoforms (*Lac1, Lac2* and *Lac3*: EC 1.10.3.2), MAP kinase (*Gmk1*: EC 2.7.11.24), endo-β-1,4-xylanase (*Xyl*: EC 3.2.1.8), β-1,3-exoglucanase (*Exo*: EC 3.2.1.58) and gentisate 1–2 dioxygenase-like (*Gdo*: EC 1.13.11.4). *18S RNA* was also quantified as a global marker of the transcription profile. The host wheat genes were glutathione-*S*-transferase (*Gst*: EC 2.5.1.18), enolase (*Eno*: EC 4.2.1.11) and cinnamyl alcohol dehydrogenase (*Cin*: EC 1.1.1.195).

The primer sets for *Ggt* genes gave amplification with fungal samples, but not with bacterial or wheat samples, and the amplifications of *Gst, Eno* and *Cin* genes were observed only with wheat samples. Complete removal of DNA was confirmed by the absence of the amplification of introns.

The amplification efficiencies of all primer pairs were optimized with serially diluted DNA or cDNA from adequate species as templates (Table 1), and were at least 79% and often close to 90% for most of the genes with r^2 values higher than 0.850. The good runs of the qPCRs for each primer set were also assessed by qPCR on serial dilutions of the mixture of cDNA from *Ggt* and Pf29Arp-inoculated roots at days 4, 7 and 10 and *Wn1* and *Wn2* external control normalizer RNAs as templates (Table 1). No influence of nontarget DNA on target DNA quantification was shown (Table 1).

The expression levels of the transcripts were calculated from the C_T values. First, for each quantified transcript (candidate genes and also *Wn1* and *Wn2* external controls), the value '1' was assigned to the lowest C_T value, namely the 'reference sample' with the highest transcript level. Second, for each transcript, the C_T values of the other samples were transformed as a relative gene expression value according to: $2^{-(CT,\text{sample} - CT,\text{'reference sample'})}$. Then, the *Wn1* and *Wn2* genes were used as exogenous controls. For this, geNorm software relies on the principle that the transcription ratio of the two reference genes is identical in all samples, regardless of experimental influences (Vandesompele *et al*., 2002). It provides an accurate gene expression normalization factor of qPCR data calculated for each sample and based on the geometric average of the transformed *Wn1* and *Wn2* C_T. For each candidate gene and each sample, the ratio between the relative expression value and this normalization factor gave the corrected expression level of the gene. Finally, each *Ggt* transcript level was corrected by the *Ggt* DNA quantified in the sample with *18S DNA* qPCR (*Ggt* 18S DNA/total DNA ratio). Each wheat transcript level was corrected by the wheat DNA/total DNA ratio, the wheat DNA being calculated as the difference between total DNA and (*Ggt* and Pf29Arp) DNA.

Statistical analyses

To satisfy the assumption of homogeneity of variance, a $\log_{10}$ transformation was applied to root symptoms and real-time qPCR data for Pf29Arp and *Ggt* DNA quantifications.

For root notations, the experiment was designed as one fixed factor with six states [two treatments (noninoculated and inoculated roots by Pf29Arp), three sampling times (4, 7 and 10 days) and one randomized block (three biological repetitions)]. The effect of Pf29Arp on take-all development was subjected to a mixed model analysis of variance.

Each gene level was subjected to a separate analysis of variance (ANOVA) using the ANOVA procedure of R statistical analysis software version 2.9.2. A Shapiro normality test of residuals was also performed.

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REFERENCES

Avrova, A.O., Venter, E., Birch, P.R.J. and Whisson, S.C. (2003) Profiling and quantifying differential gene transcription in *Phytophthora infestans* prior to and during the early stages of potato infection. *Fungal Genet. Biol.* **40**, 4–14.

Barret, M., Frey-Klett, P., Boutin, M., Guillerm-Erckelboudt, A.-Y., Martin, F., Guillot, L. and Sarniguet, A. (2009a) The plant pathogenic fungus *Gaeumannomyces graminis* var. *tritici* improves bacterial growth and triggers early gene regulations in the biocontrol strain *Pseudomonas fluorescens* Pf29Arp. *New Phytol.* **181**, 435–447.

Barret, M., Frey-Klett, P., Guillerm-Erckelboudt, A.-Y., Boutin, M., Guernec, G. and Sarniguet, A. (2009b) Effect of wheat roots infected with the pathogenic fungus *Gaeumannomyces graminis* var. *tritici* on gene expression of the biocontrol bacterium *Pseudomonas fluorescens* Pf29Arp. *Mol. Plant–Microbe Interact.* **22**, 1611–1623.

Belien, T., Campenhout, S.V., Robben, J. and Volckaert, G. (2006) Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems. *Mol. Plant–Microbe Interact.* **19**, 1072–1081.

Berruyer, R., Poussier, S., Kankanala, P., Mosquera, G. and Valent, B. (2006) Quantitative and qualitative influence of inoculation methods on in planta growth of rice blast fungus. *Phytopathology*, **96**, 346–355.

Blanco, F., Salinas, P., Cecchini, N.M., Jordana, X., Van Hummelen, P., Alvarez, M.E. and Holuigue, L. (2009) Early genomic response to salicylic acid in Arabidopsis. *Plant Mol. Biol.* **70**, 79–102.

de Boer, W., Verheggen, P., Gunnewiek, P.J.A., Kowalchuk, G.A. and van Veen, J.A. (2003) Microbial community composition affects soil fungistasis. *Appl. Environ. Microbiol.* **69**, 835–844.

Bolter, C., Brammall, R.A., Cohen, R. and Lazarovits, G. (1993) Glutathione alterations in melon and tomato roots following treatment with chemicals which induce disease resistance to *Fusarium* wilt. *Physiol. Mol. Plant Pathol.* **42**, 321–336.

Chao, S., Lazo, G.R., You, F., Crossman, C.C., Hummel, D.D., Lui, N., Laudencia-Chingcuanco, D., Anderson, J.A., Close, T.J., Dubcovsky, J., Gill, B.S., Gill, K.S., Gustafson, J.P., Kianian, S.F., Lapitan, N.L.V., Nguyen, H.T., Sorrells, M.E., McGuire, P.E., Qualset, C.O. and Anderson, O.D. (2006) Use of large-scale Triticeae expressed sequence tag resource to reveal gene expression profiles in hexaploid wheat (*Triticum aestivum* L.). *Genome*, **49**, 531–544.

Chapon, A., Guillerm, A.-Y., Delalande, L., Lebreton, L. and Sarniguet, A. (2002) Dominant colonisation of wheat roots by *Pseudomonas fluorescens* Pf29A and selection of the indigenous microflora in the presence of the take-all fungus. *Eur. J. Plant Pathol.* **108**, 449–459.

Chapon, A., Boutin, M., Rimé, D., Delalande, L., Guillerm, A.-Y. and Sarniguet, A. (2003) Direct and specific assessment of colonisation of wheat rhizoplane by *Pseudomonas fluorescens* Pf29A. *Eur. J. Plant Pathol.* **109**, 61–70.

Compant, S., Duffy, B., Nowak, J., Clément, C. and Ait Barka, E. (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* **71**, 4951–4959.

Crowe, J.D. and Olsson, S. (2001) Induction of laccase activity in *Rhizoctonia solani* by antagonistic *Pseudomonas fluorescens* strains and a range of chemical treatments. *Appl. Environ. Microbiol.* **67**, 2088–2094.

Daval, S., Lebreton, L., Gazengel, K., Guillerm-Erckelboudt, A.Y. and Sarniguet, A. (2010) Genetic evidence for differentiation of *Gaeumannomyces graminis* var. *tritici* into two major groups. *Plant Pathol.* **59**, 165–178.

De Vleesschauwer, D., Djavaheri, M., Bakker, P.A. and Höfte, M. (2008) *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. *Plant Physiol.* **148**, 1996–2012.

Deveau, A., Palin, B., Delaruelle, C., Peter, M., Kohler, A., Pierrat, J.C., Sarniguet, A., Garbaye, J., Martin, F. and Frey-Klett, P. (2007) The mycorrhiza helper *Pseudomonas fluorescens* BBc6R8 has a specific priming effect on the growth, morphology and gene expression of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *New Phytol.* **175**, 743–755.

Dodge, A.G. and Wackett, L.P. (2005) Metabolism of bismuth subsalicylate and intracellular accumulation of bismuth by *Fusarium* sp. strain BI. *Appl. Environ. Microbiol.* **71**, 876–882.

Dori, S., Solel, Z. and Barash, I. (1995) Cell wall-degrading enzymes produced by *Gaeumannomyces graminis* var. *tritici in vitro* and *in vivo*. *Physiol. Mol. Plant Pathol.* **46**, 189–198.

Dufresne, M. and Osbourn, A.E. (2001) Definition of tissue-specific and general requirements for plant infection in a phytopathogenic fungus. *Mol. Plant–Microbe Interact.* **14**, 300–307.

Edens, W.A., Goins, T.Q., Dooley, D. and Henson, J.M. (1999) Purification and characterization of a secreted laccase of *Gaeumannomyces graminis* var. *tritici*. *Appl. Environ. Microbiol.* **63**, 3071–3074.

Guilleroux, M. and Osbourn, A. (2004) Gene expression during infection of wheat roots by the 'take-all' fungus *Gaeumannomyces graminis*. *Mol. Plant Pathol.* **5**, 203–216.

Haas, D. and Défago, G. (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* **3**, 307–319.

Hatsch, D., Phalip, V., Petkovski, E. and Jeltsch, J.-M. (2006) *Fusarium graminearum* on plant cell wall: no fewer than 30 xylanase genes transcribed. *Biochem. Biophys. Res. Commun.* **345**, 959–966.

Jantasuriyarat, C., Gowda, M., Haller, K., Hatfield, J., Lu, G.D., Stahlberg, E., Zhou, B., Li, H.M., Kim, H.R., Yu, Y.S., Dean, R.A., Wing, R.A., Soderlund, C. and Wang, G. (2005) Large-scale identification of expressed sequence tags involved in rice and rice blast fungus interaction. *Plant Physiol.* **138**, 105–115.

Kang, Z., Huang, L. and Buchenauer, H. (2000) Cytochemistry of cell wall component alterations in wheat roots infected by *Gaeumannomyces graminis* var. *tritici*. *J. Plant Dis. Protect.* **107**, 337–351.

Karlsson, M., Hietala, A.M., Kvaalen, H., Solheim, H., Olson, A., Stenlid, J. and Fossdal, C.G. (2007) Quantification of host and pathogen DNA and RNA transcripts in the interaction of Norway spruce with *Heterobasidion parviporum*. *Physiol. Mol. Plant Pathol.* **70**, 99–109.

Kim, B.-R., Nam, H.-Y., Kim, S.-U., Kim, S.-I. and Chang, Y.-J. (2003) Normalization of reverse transcription quantitative-PCR with housekeeping genes in rice. *Biotechnol. Lett.* **25**, 1869–1872.

Komives, T., Gullner, G. and Kiraly, Z. (1998) Role of glutathione and glutathione-related enzymes in response of plants to environmental stress. *Ann. NY Acad. Sci.* **851**, 251–258.

Kramer, B., Thines, E. and Foster, A.J. (2009) MAP kinase signalling pathway components and targets conserved between the distantly related plant pathogenic fungi *Mycosphaerella graminicola* and *Magnaporthe grisea*. *Fungal Genet. Biol.* **46**, 667–681.

Kruger, W.M., Pritsch, C., Chao, S. and Muehlbauer, G.J. (2002) Functional and comparative bioinformatic analysis of expressed genes from wheat spikes infected with *Fusarium graminearum*. *Mol. Plant–Microbe Interact.* **15**, 445–455.

Lamb, C. and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 251–275.

Lengeler, K.B., Davidson, R.C., D'souza, C., Harashima, T., Shen, W.C., Wang, P., Pan, X., Waugh, M. and Heitman, J. (2000) Signal transduction cascades regulating fungal development and virulence. *Microbiol. Mol. Biol. Rev.* **64**, 746–785.

Litvintseva, A. and Henson, J.M. (2002) Cloning, characterization, and transcription of three laccase genes from *Gaeumannomyces graminis* var. *tritici*, the take-all fungus. *Appl. Environ. Microbiol.* **68**, 1305–1311.

Liu, Z.L. and Slininger, P.J. (2007) Universal external RNA controls for microbial gene expression analysis using microarray and qRT-PCR. *J. Microbiol. Methods*, **68**, 486–496.

Mauch, F. and Dudler, R. (1993) Differential induction of distinct glutathione-S-transferases of wheat by xenobiotics and by pathogen attack. *Plant Physiol.* **102**, 1193–1201.

Medeiros, F.C.L., Resende, M.L.V., Medeiros, F.H.V., Zhang, H.M. and Paré, P.W. (2010) Defense gene expression induced by a coffee-leaf extract formulation in tomato. *Physiol. Mol. Plant Pathol.* **74**, 175–183.

Miché, L., Battistoni, F., Gemmer, S., Belghazi, M. and Reinhold-Hurek, B. (2006) Upregulation of jasmonate-inducible defense proteins and differential colonization of roots of *Oryza sativa* cultivars with the endophyte *Azoarcus* sp. *Mol. Plant–Microbe Interact.* **19**, 502–511.

Minerdi, D., Moretti, M., Gilardi, G., Barberio, C., Gullino, M.L. and Garibaldi, A. (2008) Bacterial ectosymbionts and virulence silencing in a *Fusarium oxysporum* strain. *Environ. Microbiol.* **10**, 1725–1741.

Olivier, R.P., Farman, M.L., Jones, J.D.G. and Hammond-Kosack, K.E. (1993) Use of fungal transformants expressing β-glucuronidase activity to detect infection and measure hyphal biomass in infected plant tissues. *Mol. Plant–Microbe Interact.* **6**, 521–525.

O'Sullivan, D.J. and O'Gara, F. (1992) Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiol. Rev.* **56**, 662–676.

Pearson, V. (1974) Virulence and cellulolytic enzyme activity of isolates of *Gaeumannomyces graminis*. *Trans. Br. Mycol. Soc.* **63**, 199–202.

Raaijmakers, J.M., Paulitz, T.C., Steinberg, C., Alabouvette, C. and Moënne-Loccoz, Y. (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil*, **321**, 341–361.

Rasmussen, R. (2001) Quantification on the LightCycler. In: *Rapid Cycle Real-Time PCR, Methods and Applications* (Meuer, S., Wittwer, C. and Nakagawara, K., eds), pp. 21–34. Heidelberg: Springer Press.

Rezzonico, F., Binder, C., Défago, G. and Moënne-Loccoz, Y. (2005) The type III secretion system of biocontrol *Pseudomonas fluorescens* KD targets the phytopathogenic chromista *Pythium ultimum* and promotes cucumber protection. *Mol. Plant–Microbe Interact.* **18**, 991–1001.

Sabater-Muñoz, B., Legeai, F., Rispe, C., Bonhomme, J., Dearden, P., Dossat, C., Duclert, A., Gauthier, J.-P., Giblot Ducray, D., Hunter, W., Dang, P., Kambhampati, S., Martinez-Torres, D., Cortes, T., Moya, A., Nakabachi, A., Philippe, C., Prunier-Leterme, N., Rahbé, Y., Simon, J.-C., Stern, D.L., Wincker, P. and Tagu, D. (2006) Large-scale gene discovery in the pea aphid *Acyrthosiphon pisum* (Hemiptera). *Genome Biol.* **7**, R21-1–R21-11.

Sari, E., Hetebarian, H.R. and Aminian, H. (2007) The effects of *Bacillus pumilus*, isolated from wheat rhizosphere, on resistance in wheat seedling roots against the take-all fungus, *Gaeumannomyces graminis* var. *tritici*. *J. Phytopathol.* **155**, 720–727.

Sari, E., Hetebarian, H.R. and Aminian, H. (2008) Effects of *Pseudomonas fluorescens* CHAO on the resistance of wheat seedling roots to the take-all fungus *Gaeumannomyces graminis* var. *tritici*. *Plant Prod. Sci.* **11**, 298–306.

Sarniguet, A., Boutin, M., Guillerm-Erckelboudt, A.-Y. and Barret, M. (2006) Three mechanisms for the biocontrol activity of *Pseudomonas fluorescens* Pf29A against the plant pathogenic fungus *Gaeumannomyces graminis* var. *tritici*. In: *7th International Workshop on Plant Growth Promoting Rhizobacteria*, Noordwijkerhout, the Netherlands, 28 May–2 June 2006 (Bakker, P., Raaijmakers, J., Bloemberg, G., Höfte, M. and Lemanceau, P., eds), p. 25.

Schrey, S.D., Schelhammer, M., Ecke, M., Hampp, R. and Tarkka, M.T. (2005) Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria*. *New Phytol.* **168**, 205–216.

Shoresh, M., Harman, G.E. and Mastouri, F. (2010) Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* **48**, 21–43.

Southerton, S.G., Osbourn, A.E., Dow, J.M. and Daniels, M.J. (1993) Two xylanases from *Gaeumannomyces graminis* with identical N-terminal amino acid sequence. *Physiol. Mol. Plant Pathol.* **42**, 97–107.

Tarkka, M.T., Sarniguet, A. and Frey-Klett, P. (2009) Inter-kingdom encounters: recent advances in molecular bacterium–fungus interactions. *Curr. Genet.* **55**, 233–243.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative qPCR data by geometric averaging of multiple internal controls. *Genome Biol.* **3**, 1–12.

Van Loon, L.C., Bakker, P.A.H. and Pieters, C.M. (1998) Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* **36**, 453–483.

Van Wees, S.C., Pieterse, C.M., Trijssenaar, A., Van't Westende, Y.A., Hartog, F. and Van Loon, L.C. (1997) Differential induction of systemic resistance in Arabidopsis by biocontrol bacteria. *Mol. Plant–Microbe Interact.* **10**, 716–724.

Velasquez-Cedeno, M.A., Farnet, A.M., Ferre, E. and Savoie, J.M. (2004) Variations of lignocellulosic activities in dual cultures of *Pleurotus ostreatus* and *Trichoderma longibrachiatum* on unsterilized wheat straw. *Mycologia*, **96**, 712–719.

Willocquet, L., Lebreton, L., Sarniguet, A. and Lucas, P. (2008) Quantification of within-season focal spread of wheat take-all in relation to pathogen genotype and host spatial distribution. *Plant Pathol.* **57**, 906–915.

Wu, S.-C., Halley, J.E., Luttig, C., Fernekes, L.M., Gutierrez-Sanchez, G., Darvill, A.G. and Albersheim, P. (2006) Identification of an endo-β-1,4-D-xylanase from *Magnaporthe grisea* by gene knockout analysis, purification and heterologous expression. *Appl. Environ. Microbiol.* **72**, 986–993.

Yu, Y., Kang, Z., Buchenauer, H. and Huang, L. (2009) Purification and characterization of a novel extracellular β-1,3-glucanase complex (Glu*Ggt*) secreted by *Gaeumannomyces graminis* var. *tritici*. *World J. Microbiol. Biotechnol.* **25**, 2179–2186.

Zhang, H., Hong, Y.Z., Xiao, Y.Z., Yuan, J., Tu, X.M. and Zhang, X.Q. (2006) Efficient production of laccases by *Trametes* sp AH28-2 in cocultivation with a *Trichoderma* strain. *Appl. Microbiol. Biotechnol.* **73**, 89–94.

Zhao, J., Buchwaldt, L., Rimmer, S.R., Sharpe, A., McGregor, L., Bekkaoui, D. and Hegedus, D. (2009) Patterns of differential gene expression in *Brassica napus* cultivars infected with *Sclerotinia sclerotiorum*. *Mol. Plant Pathol.* **10**, 635–649.

Zhao, X., Mehrabi, R. and Xu, J.R. (2007) Mitogen-activated protein kinase pathways and fungal pathogenesis. *Eukaryot. Cell*, **6**, 1701–1714.