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.

**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 nutrients, 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 *Gaumannomyces 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; Shores 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 transcription 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 *Gaumannomyces 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 antagonist 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 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
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 log10 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 absence of Pf29Arp.
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,
Table 1  Efficiency of quantitative polymerase chain reactions (qPCRs).

| Gene target | Organism                  | Standard curve established with cDNA (from aphid*, bacteri†, 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 |
|-------------|---------------------------|-----------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| Wn1         | Acyrthosiphon pisum       | Slope (E (%), r²)                                                                                                    | Slope (E (%), r²)                                                                                   |
| Wn2         | Acyrthosiphon pisum       | –3.68* 87 0.998                                                                                                      | –3.40 97 0.977                                                                                     |
| Pf29A-DP    | Pseudomonas fluorescens   | –3.39 97 0.846                                                                                                      | –3.35 92 0.995                                                                                     |
| 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 | –3.71 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-B-1,4-xylanase; Exo, B-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 $E = 10^{(-1/slope)} - 1$ (Rasmussen, 2001). The correlations ($r²$) between the concentration of target DNA or cDNA and the observed C; 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...
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 P129Arp 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 gnotobiotic 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 P129Arp 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, P129Arp 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 P29Arp 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 P29Arp 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 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 P29Arp 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 P29Arp, 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 P29Arp 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 Azorarcs 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 P29Arp. 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 P29Arp. 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-mm³ 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, 10⁶ 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 and mortar (Microvision Instruments, Evry, France). Disease incidence was manually designed using Primer 3. Amplification reactions were performed in a volume of 50 μL containing 1.8 mM MgCl₂, 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, Wn177 and Wn277, 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 RNase free DNase, RNA purity and quality were checked 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 (Berrey *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 phosphatide 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′-TAATAAGCTACTAGTAG-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 MgCl₂, 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, *Wn177* and *Wn277*, 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 RNase free 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 MgCl₂, 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) |
|--------------|--------|----------|----------------|---------------|--------------------|----------------------------|
| Wn177        | Sabater-Muñoz et al. (2006) | Acyrthosiphon pisum | 5′-TAATACGACTCACTATAGGGGAAGCCCAACAAATTGACG-3′ | 5′-GCCGTTCCTCCGGAGGATTTAT-3′ | 336 | 50 |
| Wn277        | Sabater-Muñoz et al. (2006) | Acyrthosiphon pisum | 5′-TAATACGACTCACTATAGGGGAAGCCCAACAAATTGACG-3′ | 5′-GCCGTTCCTCCGGAGGATTTAT-3′ | 336 | 50 |
| Wn1          | Sabater-Muñoz et al. (2006) | Acyrthosiphon pisum | 5′-TCTTTTTCCTGGCAGTGTT-3′ | 5′-GCCGTTCCTCCGGAGGATTTAT-3′ | 336 | 50 |
| Wn2          | Sabater-Muñoz et al. (2006) | Acyrthosiphon pisum | 5′-TCTTTTTCCTGGCAGTGTT-3′ | 5′-GCCGTTCCTCCGGAGGATTTAT-3′ | 336 | 50 |
| Pf29A-DP     | AF360119 | Pseudomonas fluorescens | 5′-GCAAATGATGATCAATGAGG-3′ | 5′-GAAATCCTCGGCTGTT-3′ | 107 | 60 |
| 18S rDNA     | FJ771002 | Gaeumannomyces graminis var. tritici | 5′-CGAATCTCGTGGTTAATGGG-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |
| 18S rRNA     | FJ771002 | Gaeumannomyces graminis var. tritici | 5′-CGAATCTCGTGGTTAATGGG-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |
| Lac1         | AF1417685 | Gaeumannomyces graminis var. tritici | 5′-CTGTCCTGAGAATCTGTC-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |
| Lac2         | AF1417686 | Gaeumannomyces graminis var. tritici | 5′-CTGTCCTGAGAATCTGTC-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |
| Lac3         | AF1417687 | Gaeumannomyces graminis var. tritici | 5′-CTGTCCTGAGAATCTGTC-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |
| Xyl          | AF1249160 | Gaeumannomyces graminis var. tritici | 5′-CTGTCCTGAGAATCTGTC-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |
| Exp          | CF554536 | Gaeumannomyces graminis var. tritici | 5′-CTGTCCTGAGAATCTGTC-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |
| Gdo          | CF717712 | Gaeumannomyces graminis var. tritici | 5′-CTGTCCTGAGAATCTGTC-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |
| Gst          | CF554506 | Wheat (Triticum aestivum) | 5′-CTGTCCTGAGAATCTGTC-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |
| Eno          | CF554506 | Wheat (Triticum aestivum) | 5′-CTGTCCTGAGAATCTGTC-3′ | 5′-ATTGATCCTCGGCTGTT-3′ | 107 | 60 |

*Wn177, amplonc of the Wunen expressed sequence tag (EST) 3′ region used as in vitro transcription template; Wn277, amplonc 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, lacase 1; Lac2, lacase 2; Lac3, lacase 3; Gmk1, mitogen-activated protein (MAP) kinase; Xyl, endo-β-1,4-xylanase; Exp, β-1,3-exoglucanase; Gdo, gentisate 1–2 dioxygenase-like; Gst, glutathione S-transferase; Enol, 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, \(1 \times \) ImPromII reaction buffer, 3 mM MgCl₂, 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 \(\times\) 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 \((r² > 0.846)\) 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 \(\left(C\right)\) 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 \((\left.C\right)\) value) was used to calculate the total DNA amplified. The \(C\) values determined were plotted against the logarithm of their known initial concentrations, the standard curve generated by linear regression through these points linking observed \(C\) 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 \(C\) values. The amounts of bacterial and fungal DNA were expressed as \(log_{10}\)(number of amplified fragment copies/μg 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 isofoms \((Lac1, Lac2\) and \(Lac3\): EC 1.10.3.2), MAP kinase \((Gmk1): EC 2.7.11.24), endo-\(β\)-1,4-xylanase \((XYf): EC 3.2.1.8), \(β\)-1,3-exoglucanase \((Exo): EC 3.2.1.58) and gentisate 1–2 dioxygenase-lyase \((Gdo): EC 1.13.11.4). \(18S\) \(rDNA\) 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 \((Eon): EC 4.2.1.11\) and cinnamyl alcohol dehydrogenase \((Cin): EC 1.1.1.195) at 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 primer sets for \(Ggt\) genes gave amplification with fungal samples, but not with bacterial or wheat samples, and the amplifications of \(Gst\), \(Eon\) 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 \(r²\) 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\textsubscript{T} values. First, for each quantified transcript (candidate genes and also \textit{Wn1} and \textit{Wn2} external controls), the value ‘1’ was assigned to the lowest \textit{C\textsubscript{T}} value, namely the ‘reference sample’ with the highest transcript level. Second, for each transcript, the \textit{C\textsubscript{T}} values of the other samples were transformed as a relative gene expression value according to: \(2^{-\Delta\text{C\textsubscript{T}}(\text{sample} - \text{C\textsubscript{T}}(\text{reference sample}))}\). Then, the \textit{Wn1} and \textit{Wn2} genes were used as exogenous controls. For this, geNorm software relies on the principle that the transcript 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 \textit{Wn1} and \textit{Wn2} \textit{C\textsubscript{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 \textit{Ggt} transcript level was corrected by the \textit{Ggt} DNA quantified in the sample with \textit{18S} DNA qPCR (\textit{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 (\textit{Ggt} and Pf29Arp) DNA.

Statistical analyses

To satisfy the assumption of homogeneity of variance, a log\textsubscript{10} transformation was applied to root symptoms and real-time qPCR data for Pf29Arp and \textit{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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