Induced systemic resistance responses in perennial ryegrass against *Magnaporthe oryzae* elicited by semi-purified surfactin lipopeptides and live cells of *Bacillus amyloliquefaciens*

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SUMMARY

The suppressive ability of several strains of cyclic lipopeptide-producing *Bacillus* rhizobacteria to grey leaf spot disease caused by *Magnaporthe oryzae* has been documented previously; however, the underlying mechanism(s) involved in the induced systemic resistance (ISR) activity in perennial ryegrass (*Lolium perenne* L.) remains unknown. Root-drench application of solid-phase extraction (SPE)-enriched surfactin and live cells of mutant *Bacillus amyloliquefaciens* strain FZB42-AK3 (produces surfactin, but not bacillomycin D and fengycin) significantly reduced disease incidence and severity on perennial ryegrass. The application of the treatments revealed a pronounced multilayered ISR defence response activation via timely and enhanced accumulation of hydrogen peroxide (H_2O_2), elevated cell wall/apoplastic peroxidase activity, and deposition of callose and phenolic/polyphenolic compounds underneath the fungal appressoria in naïve leaves, which was significantly more intense in treated plants than in mock-treated controls. Moreover, a hypersensitive response (HR)-type reaction and enhanced expression of *LpPrx* (Prx, peroxidase), *LpOXO4* (OXO, oxalate oxidase), *LpPAL* (PAL, phenylalanine ammonia lyase), *LpLOXa* (LOX, lipoxygenase), *LpTHb* (putative defensin) and *LpDEFa* (DEFa, putative defensin) in perennial ryegrass were associated with SPE-enriched surfactin and live AK3 cell treatments, acting as a second layer of defence when pre-invasive defence responses failed. The results indicate that ISR activity following surfactin perception may sensitize H_2O_2 -mediated defence responses, thereby providing perennial ryegrass with enhanced protection against *M. oryzae*.

Keywords: H_2O_2 accumulation, hypersensitive response, peroxidase activity, real-time RT-PCR, solid-phase extraction (SPE).

INTRODUCTION

Perennial ryegrass is an important forage and turf-type grass because of its superior nutritional and agronomic qualities (Ran

et al., 2007; Uddin et al., 2003). Recently, grey leaf spot, caused by *Magnaporthe oryzae*, has emerged as one of the most perilous diseases of this turfgrass species (Rahman et al., 2014). As a result of heavy reliance on chemical fungicides to control the disease, fungicide-resistant *M. oryzae* strains have already emerged (Vincelli and Dixon, 2002), and non-target effects on beneficial microbes and the possibility of environmental quality deterioration are of serious concern (Pérez-García et al., 2011). The use of beneficial microbes, together with host resistance, holds great potential as an environmentally friendly approach in the management of plant diseases (Pérez-García et al., 2011).

Some of the most common responses of induced host resistance in plants following pathogen recognition include cell wall fortification, such as callose deposition in cell wall appositions (CWAs), and localized accumulation of antimicrobial compounds (Ahn et al., 2007; Jones and Dangl, 2006; Pieterse et al., 2009). Such local reactions are often followed by a burst of reactive oxygen species (ROS), e.g. hydrogen peroxide (H_2O_2), resulting in the onset of programmed cell death (PCD), referred to as a 'hypersensitive response' (HR), leading to systemic resistance (Durrant and Dong, 2004; Jones and Dangl, 2006). Regardless of its origin, H_2O_2 is an important defence signalling molecule and displays direct toxicity towards invading microbial pathogens (Torres, 2010). In addition, pathogen-induced systemic resistance, known as systemic acquired resistance (SAR), is commonly associated with an enhanced level of endogenous salicylic acid (SA) and *pathogenesis-related 1* (*PR-1*), whereas beneficial microorganisms trigger induced systemic resistance (ISR), which is often regulated by jasmonic acid/ethylene (JA/ET)-dependent signalling pathways with concomitant induction of *PDF1.2* (*plant defensin 1.2*) in *Arabidopsis* (Kachroo and Kachroo, 2009; Pieterse et al., 2009). Although rhizobacteria-mediated ISR predominantly involves an SA-independent JA/ET-mediated defence response, some rhizobacteria may trigger the SA-dependent signalling pathway (De Vleeschauwer et al., 2006). A variety of bacterial determinants contributing to ISR-eliciting activity have been identified, including flagella, lipopolysaccharides, quorum-sensing molecules, volatile organic compounds (VOCs) and cyclic lipopeptides (CLPs) (Jourdan et al., 2009; Pieterse et al., 2014).

CLPs, namely surfactin, iturin and fengycin family CLPs, are generated by multiple species of *Bacillus* with an array of

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biological activity (Raaijmakers *et al.*, 2010). The surfactin family encompasses heptapeptides interlinked with β -hydroxy fatty acid chains with a length of 12–16 carbon atoms to form a cyclic lactone ring structure, and exhibit strong antiviral and antibacterial, but no marked antifungal, activity (Henry *et al.*, 2011). Surfactin and fengycin, but not iturin, have also been reported to elicit ISR in plants against fungal pathogens, albeit exhibiting differential preference towards different plant cell types (Ongena and Jacques, 2008). The plant root-colonizing bacterium *B. amyloliquefaciens* strain FZB42 (Fan *et al.*, 2012) is a naturally occurring isolate capable of stimulating plant growth (Idris *et al.*, 2007). Furthermore, the strain produces all three families of lipopeptides, namely surfactin, bacillomycin D (iturin family member) and fengycin (Koumoutsis *et al.*, 2004).

Studies involving ISR activity in turfgrass species are very scarce. Whether any ISR activity in perennial ryegrass could be elicited following the application of CLPs is yet to be documented. The primary objective of this study was to investigate the role of rhizobacteria-originating surfactin in suppressing grey leaf spot disease in perennial ryegrass. ISR activity in perennial ryegrass to suppress *M. oryzae* infection was studied through the application of a mutant *B. amyloliquefaciens* FZB42-AK3 strain, which is deficient in the production of bacillomycin D and fengycin (and hence only produces surfactin) (Koumoutsis *et al.*, 2004), and a semi-purified (solid-phase extraction, SPE) surfactin preparation.

RESULTS

Suppression of grey leaf spot disease by *B. amyloliquefaciens* live cells and SPE-enriched surfactin (SPE-surfactin) lipopeptide

The purpose of this experiment was to evaluate the potential of root-drench application of live *B. amyloliquefaciens* FZB42-AK3 cells and SPE-surfactin in suppressing grey leaf spot disease of perennial ryegrass. In order to analyse the disease lesion severity, grey leaf spot lesion types were divided into four groups based on a previous report (Table 1) (Curley *et al.*, 2005). The appli-

Table 1 Grey leaf spot lesion types on perennial ryegrass leaf blades at 5 days post-inoculation with *Magnaporthe oryzae* (Curley *et al.*, 2005).

Type*	Description
I	Dark brown to black, non-sporulating lesions <1.0–3.0 mm in length
II	Dark brown to black lesion with central, often sporulating, necrotic area, 1.0–3.0 mm in length
III	Circular or small diamond-shaped lesions with dark brown to black borders with greyish central sporulating areas
IV	Completely unbordered, large expanding dark brown lesions with or without sporulating area with chlorotic haloes

*Lesion types were numbered according to the severity, type IV being the most severe (causing leaf discoloration and blighting) and types I and II being the least severe.

cation of live cells of wild-type (wt) *B. amyloliquefaciens* FZB42, $\Delta bmyA\Delta fenA$ *B. amyloliquefaciens* FZB42-AK3 strain (bacillomycin D- and fengycin-deficient mutant) and SPE-surfactin (500 μ g/mL) significantly reduced both the disease incidence (DI) and disease severity (DS) of grey leaf spot relative to mock-treated control plants ($P < 0.05$) (Fig. 1a). Subsequent bioassays revealed that SPE-surfactin, when applied at 250 μ g/mL (241.3 μ M) and 500 μ g/mL (488.62 μ M), but not at 100 μ g/mL (96.52 μ M), concentrations significantly suppressed grey leaf spot disease ($P < 0.05$) (Fig. 1b). However, all four different lesion types (Fig. 1c) observed in treated plants differed significantly from those of mock-treated control plants ($P < 0.05$) (Fig. 1d). Lesion type IV (most severe susceptible reaction causing leaf discoloration and blighting) was more prevalent (73.9%) in mock-treated control plants than in AK3- and SPE-surfactin-treated plants (30.4% and 20.6%, respectively) (Fig. 1d). In the last two treatments, lesion type III was the most predominant symptom type observed (35.5% and 32.5%, respectively) with type I being the least prevalent (11.8% and 16.9%, respectively) (Fig. 1d). However, there were no significant differences between wt-FZB42- and mutant AK3-treated plants in the lesion type assay (data not shown).

We have demonstrated previously that live bacterial cells and enriched lipopeptide extracts from *B. amyloliquefaciens* FZB42 inhibit *M. oryzae* mycelial growth and spore germination (Rahman and Uddin, 2010). To rule out the possibility that disease suppression was caused by a direct effect from the possible migration of root-inoculated bacteria, foliar tissues were analysed for AK3 bacterial cell count. The presence of AK3 colonies was not detected in randomly collected leaf samples from three independent experiments, with a reproducible detection limit of 10^2 colony-forming units (CFU)/g tissues, suggesting that bacterial colonization remained confined to the rhizosphere. However, the rhizosphere concentrations of FZB42 and AK3 cells were found to be $(6.1 \pm 2.1) \times 10^6$ and $(5.3 \pm 3.2) \times 10^6$ CFU/g of root fresh weight in 5-week-old plants.

Cytological analysis of the perennial ryegrass defence response against *M. oryzae*

3,3'-Diaminobenzidine (DAB) staining

To gain an insight into the mechanism(s) associated with AK3- and SPE-surfactin-mediated ISR, the generation of pathogenesis-related H_2O_2 was analysed using DAB staining (Thordal-Christensen *et al.*, 1997). Consistent differences in DAB reaction types between treated and mock-treated control plants were observed from 36 h post-inoculation (hpi) onwards (Fig. S1, see Supporting Information). DAB-facilitated H_2O_2 detection was confirmed by 10 mM ascorbate treatment, which abolished the brown colour (Thordal-Christensen *et al.*, 1997). Starting at 36 hpi, epidermal cells of leaf blades were found to respond to

Fig. 1 Effects of *Bacillus amyloliquefaciens* live cells and solid-phase extraction (SPE)-enriched surfactin treatment as root-drench method on grey leaf spot disease incidence (DI) and disease severity (DS) development on perennial ryegrass. (a) DI and DS levels [disease score (%)] in plants treated with live cells of wild-type *B. amyloliquefaciens* FZB42 or $\Delta bmyA\Delta fenA$ *B. amyloliquefaciens* FZB42-AK3 (AK3) or 500 µg/mL of SPE-enriched surfactin. (b) DI and DS levels in plants treated with different concentrations (µg/mL) of SPE-enriched surfactin (Srf). (c) Disease lesion types on the symptomatic leaf blades 5 days post-inoculation with *Magnaporthe oryzae* as described in Table 1. (d) Percentage of different lesion types observed on symptomatic leaf blades treated with AK3 live cells and SPE-enriched surfactin; data from one representative experiment are presented. Mock-treated control plants were treated with saline (0.85%) water. Different letters indicate significant differences from control and among treatments using Student–Newman–Keul’s test ($P < 0.05$). Each bar represents mean $\pm$ standard error for three replicates from one representative experiment. Repetition of experiments led to results very similar to those shown.

pathogen ingress through the development of various cellular reaction types that were grouped into six categories (Fig. 2a).

- Type I: successful fungal invasion with no visible cellular responses in the infected epidermal cell, or neighbouring epidermal or mesophyll cells.
- Type II: DAB-positive partial staining observed as a yellowish-brown halo surrounding the appressorial penetration point of the epidermal cell, indicating the accumulation of local H_2O_2 in CWAs.
- Type III: pale yellow or brownish staining of anticlinal cell walls of the primary invaded epidermal cell, together with successful fungal invasion of neighbouring cells, without the accumulation of any cytoplasmic vesicles or granules or browning of the whole cell.
- Type IV: DAB-positive intense browning of the infected epidermal cell, as well as neighbouring epidermal and mesophyll cells.

- Type V: DAB-positive whole-cell staining of the primary infected cell only, associated with the abrupt arrest of intracellular hypha (IH), when present, within the primary penetrated cell.
- Type VI: single-cell infection site associated with the occurrence of DAB-positive intense brownish granules in the cell cytoplasm.

Based on the type of DAB reaction, types I and III were considered to be associated with compatible interaction, whereas types IV–VI represented single- or multiple-cell HR-type interaction. At 36 hpi, control plants exhibited only the first three types of DAB reaction (I–III), with types I and III comprising the majority (>90%) of all interactions, suggesting the lack of development of any major fungal inhibitory defence responses (Fig. 2b). However, in AK3- and SPE-surfactin-treated plants, compatible interaction types (I and III) made up less than 60% of all interaction sites, whereas DAB reaction type II appeared to be the most prevalent

Fig. 2 (a) Classification of cytological perennial ryegrass–*Magnaporthe oryzae* interaction types in leaf epidermal cells following 3,3'-diaminobenzidine (DAB) staining: I, successful invasion of fungal hyphae with no visible plant cellular responses; II, DAB-positive H_2O_2 accumulation in the cell wall appositions (CWAs); III, pale yellow or brownish staining of anticlinal cell walls of the primary infected cell together with intracellular hyphae (IH) in neighbouring cells, but no detectable H_2O_2 accumulation; IV, DAB-positive browning of the primary infected and neighbouring epidermal and mesophyll cells; V, DAB-positive single whole-cell browning; VI, intense DAB-positive brownish granules in primary infected cell. Bars, 20 μ m. Ap, appressorium. (b) Frequency of different DAB-associated cellular phenotypes in treated and mock-treated perennial ryegrass leaves following inoculation with *M. oryzae*. Data from one experiment are presented. Repetition of the experiments led to very similar results as shown here. Differences in each interaction type observed between treated and mock-treated control plants were evaluated using Student–Newman–Keul's test. The presence of an asterisk indicates significant differences ($P < 0.05$) in each interaction type in treated plants relative to mock-treated plants.

type in SPE-surfactin-treated plants (Fig. 2b). By 48 hpi, type II reaction was no longer visible in mock-treated control plants with less than one-fifth of all infection sites associated with HR-type reactions (Fig. 2a, IV–VI). By contrast, DAB reaction type II was not only visible in both AK3- and SPE-surfactin-treated plants, but was also the most predominant interaction type in SPE-surfactin-treated plants at 48 hpi (Fig. 2b). Furthermore, unlike mock-treated control plants, in AK3- and SPE-surfactin-treated plants, more than one-third of all interaction types belonged to HR-type reactions (Fig. 2a, IV–VI).

Accumulation of phenolic compounds

Phenolic compounds were found to accumulate at the sites of attempted pathogen entry inside CWAs, as well as in the entire infected cells and in the adjacent neighbouring cells (Fig. 3a,b). Although autofluorescence was detectable irrespective of the treatment, significantly greater numbers of interaction sites in AK3- and SPE-surfactin-treated plants exhibited augmented deposition of phenolic compounds at 24 and 48 hpi relative to those in

mock-treated control plants ($P < 0.05$) (Fig. 3c). CWAs containing polyphenolic compounds were also detected using toluidine blue staining (Fig. 4a–c). By 48 and 72 hpi, significantly more CWAs exhibited toluidine blue-positive lignin/polyphenolic deposition in AK3- and SPE-surfactin-treated perennial ryegrass relative to those in mock-treated control plants ($P < 0.05$) (Fig. 4d). In addition, enhanced and stronger deposition of lignin/polyphenolic compounds was observed in treated plants (Fig. 4a) than in mock-treated controls (Fig. 4b). Furthermore, mock-treated control leaf blades more frequently exhibited IH by 36 hpi (Fig. 4c), indicating failure of the lignin/polyphenolic CWAs in these plants to restrict fungal penetration.

Callose deposition and IH propagation

When present in CWAs, callose deposition can either prevent the penetration of appressorial penetration pegs (Fig. 5a,b) or form a haustorial collar neck (Fig. 5c,d) when breached (Zeyen *et al.*, 2002). Callose deposition was observed regardless of the treatment type by 24 hpi (Table 2). However, both AK3 and SPE-

Fig. 3 Representative epifluorescence images of mock-treated control (a) and solid-phase extraction (SPE)-enriched surfactin-treated (b) perennial ryegrass epidermal cells with attempted penetration sites of *Magnaporthe oryzae* conidia at 24 h post-inoculation (hpi). Autofluorogenic phenolics are visible in cell wall appositions (CWAs), as well as in whole cells beneath appressoria. Treatment with AK3 live cells generated very similar images to those of the SPE-enriched surfactin treatment. Bars, 20 µm. Ap, appressorium. (c) Frequency of autofluorogenic phenolic deposition at the attempted penetration sites of *M. oryzae* conidia on leaf epidermal cells in treated and mock-treated perennial ryegrass leaves following inoculation with *M. oryzae*. Values represent the means $\pm$ standard deviation. The presence of an asterisk indicates a significant difference from mock-treated control plants using Student–Newman–Keul’s test ($P < 0.05$).

Fig. 4 Representative images of toluidine blue staining for lignin/polyphenolics (appear turquoise blue) of solid-phase extraction (SPE)-enriched surfactin-treated (a) and mock-treated control (b) perennial ryegrass epidermal cells with attempted penetration sites of *Magnaporthe oryzae* conidia at 24 h post-inoculation (hpi). (c) Penetrated lignin/polyphenolic-deposited cell wall appositions (CWAs) in which *M. oryzae* had established intracellular hyphae (IH) at 36 hpi were frequently observed in control but not treated plants. Treatment with AK3 live cells generated very similar images to those of SPE-enriched surfactin treatment. Bars, 20 µm. Ap, appressorium. (d) Frequency of lignin/polyphenolic deposition at the attempted penetration sites of *M. oryzae* conidia on leaf epidermal cells in treated and mock-treated perennial ryegrass leaves following inoculation with *M. oryzae*. Values represent the means $\pm$ standard deviation. The presence of an asterisk indicates significant difference from mock-treated control plants using Student–Newman–Keul’s test ($P < 0.05$).

surfactin treatment resulted in significantly higher proportions of callose deposition at 48 and 72 hpi relative to that in mock-treated control plants ($P < 0.05$). Furthermore, significantly higher proportions of all deposited callose were penetrated by fungal penetration pegs in mock-treated control plants (6.5% of all attempted penetration sites were non-penetrated) relative to that

in AK3- and SPE-surfactin-treated plants (15% and 31% remained non-penetrated, respectively) at 48 hpi ($P < 0.05$) (Table 2).

A detailed examination of the temporal changes in IH proliferation was visualized using KOH–aniline blue staining in *M. oryzae*-inoculated plants (Fig. 6a–h). In mock-treated control plants, a rapid growth of IH was observed by 36 hpi and, within 48 hpi,

Fig. 5 Representative images of aniline blue-stained callose deposition inside cell wall appositions (CWAs) during attempted penetration by *Magnaporthe oryzae* conidia on perennial ryegrass epidermal cells. Non-penetrated papillae showing localized deposition of callose beneath the attempted penetration sites as seen by epifluorescent microscopy with white light (a) and UV filter (b). Penetrated callose-deposited papilla on epidermal cell, as viewed by epifluorescent microscopy with white light (c) and UV filter (d), in which *M. oryzae* has established intracellular hyphae (IH). Treatment with AK3 live cells generated very similar images to those of solid-phase extraction (SPE)-enriched surfactin treatment. Bars, 20 µm. Ap, appressorium.

Table 2 Callose deposition on perennial ryegrass leaf blades at *Magnaporthe oryzae* infection sites during different time points.

Time (hpi)	Interaction sites with deposited callose* (%)		
	Mock-treated control	SPE-surfactin treated	AK3 treated
24	16.8 a ± 2.6	22.7 a ± 2.9	21.0 a ± 3.1
48	19.3 a ± 1.7	42.4 b ± 3.9	30 c ± 2.7
72	21.5 a ± 3.0	45.5 b ± 5.3	31.6 c ± 2.5
Non-penetrated‡			
48	33.7 a ± 4.9	72.7 b ± 2.6	50.4 c ± 7.8

*Data represent the mean ± standard deviation of 75–125 interaction sites at each time point with three replicates. AK3, live cells of *B. amyloliquefaciens* FZB42-AK3; SPE-surfactin, solid-phase extraction-enriched surfactin at 250 µg/mL concentration.

†Values followed by different letters within the row are significantly different according to Student–Newman–Keul's multiple range test ($P < 0.05$).

‡Percentage of callose-deposited sites that were not penetrated by a *M. oryzae* penetration peg at the designated time point.

resulted in secondary cell (secondary epidermal cell) infection (Fig. 6a,b). However, in AK3- and SPE-surfactin-treated plants, *M. oryzae* IH was contained within the primary infected cell (almost 80% of infection sites when IH was present), even at 48 hpi, and invasion into secondary cells was not observed until 60 hpi (Fig. 6f,g). Conversely, in mock-treated control plants, by 60 hpi, multiple surrounding epidermal cells from the initial focal point were invaded by IH (Fig. 6c) and, by 72 hpi, extensive proliferation of IH and massive fungal biomass encompassing multiple layers of neighbouring cells were observed (almost 80% of all infection sites) (Fig. 6d). In contrast, in AK3- and SPE-surfactin-treated plants, by 72 hpi, *M. oryzae* IH were contained within the secondary infected cells in approximately 30% and 45% of all infected sites examined, respectively (Fig. 6h).

Fig. 6 Representative images of KOH–aniline blue staining of fungal hyphae inside epidermal cells of perennial ryegrass leaves infected with *Magnaporthe oryzae* observed under an epifluorescent microscope. (a–d) Mock-treated control. (e–h) Solid-phase extraction (SPE)-enriched surfactin-treated perennial ryegrass. (a, e) 36 h post-inoculation (hpi); (b, f) 48 hpi; (c, g) 60 hpi; (d, h) 72 hpi. Treatment with AK3 live cells generated very similar images to those of SPE-enriched surfactin treatment. Bars, 20 µm. Ap, appressorium; IH, intracellular hyphae; 1°, primary infected epidermal cell; 2°, secondary infected epidermal cell.

Defence-related gene expression analysis

To investigate the resistance mechanisms at a molecular level, gene expression of selected defence-related genes, namely

Fig. 7 Expression pattern of perennial ryegrass genes [(a) *Prx*, (b) *OXO4*, (c) *PAL*, (d) *LOXa*, (e) *THb*, (f) *DEFa*] following inoculation with *Magnaporthe oryzae*. Filled white bars, mock-treated control; striped bars, solid-phase extraction (SPE)-enriched surfactin treated; filled black bars, AK3 live cell treated. Each bar represents the average relative expression ($\pm$ SD) of *PR* genes normalized to the housekeeping gene *eEF1A(s)* (stably expressed eukaryotic elongation factor 1 α) for three replicates. Fold changes were calculated by calibrating all data to the 0-h time point of a healthy control plant leaf tissue sample using the comparative threshold method ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen, 2001). Different letters indicate significant differences from mock-treated control using Student–Newman–Keul’s test ($P < 0.05$).

LpPrx (*Prx*, peroxidase), *LpOXO4* (*OXO*, oxalate oxidase), *LpPAL* (*PAL*, phenylalanine ammonia lyase), *LpLOXa* (*LOX*, lipoxygenase), *LpTHb* (putative defensin) and *LpDEFa* (*DEFa*, putative α -defensin), was analysed using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). These *PR* genes were selected on the basis of previous reports on transcriptome analysis of H_2O_2 - and pathogenic fungus-mediated induction of defence-related genes in plant pathosystems (Eaton *et al.*, 2010; Huckelhoven, 2007; Jourdan *et al.*, 2009).

An induction of *LpPrx* expression was observed in AK3- and SPE-surfactin-treated plants by 24 hpi, which was more than 2.0-fold and 3.6-fold higher, respectively, than in mock-treated control plants ($P < 0.05$) (Fig. 7a). Similarly, in AK3- and SPE-surfactin-treated plants, an increased (4.2-fold and 10.4-fold, respectively) expression level of *OXO4*, another candidate for the production of defence-related extracellular H_2O_2 in cereal plants (Molla *et al.*, 2013; Zhou *et al.*, 1998), was observed by 24 hpi (Fig. 7b). In both instances, only SPE-surfactin-treated plants exhibited significantly higher expression ($P < 0.05$) than mock-treated control plants at all times. In addition, a much stronger and significant induction of both *PAL* (Fig. 7c) and *LOXa* (Fig. 7d) genes was associated with

AK3- (7.9-fold and 6.9-fold, respectively) and SPE-surfactin- (13.5-fold and 13.2-fold, respectively) treated plants by 24 hpi, relative to that in mock-treated control plants ($P < 0.05$). However, by 72 hpi, the relative expression levels of both genes were comparable in all plants with no significant difference. Plant defensins (*PR*-12 proteins) exhibit membrane-permeabilizing ability (Edreva, 2005; Huckelhoven, 2007) and are deposited together with callose following pathogen inoculation (Jensen *et al.*, 2008). The analysis of two putative defensin genes, *LpTHb* and *LpDEFa*, indicated an elevated expression level in both AK3- (1.7-fold and 3.7-fold, respectively) and SPE-surfactin- (8.1-fold and 3.8-fold, respectively) treated plants relative to control plants within 24 hpi ($P < 0.05$) (Fig. 7e,f). Although the expression level of *LpTHb* peaked at 48 hpi, *LpDEFa* exhibited a peak expression level at 72 hpi in both AK3- and SPE-surfactin-treated plants. However, the relative expression of *LpTHb* in AK3-treated plants was found to be no different from that of mock-treated control plants at 72 hpi. In addition, in mock-treated control plants, the relative expression levels of both *LpTHb* and *LpDEFa* were higher at 72 hpi relative to earlier time points. Collectively, these gene expression analyses indicated that AK3 and SPE-surfactin treatment resulted in an

attacker-induced augmented expression of defence-related genes relative to that in mock-treated control plants.

DISCUSSION

Root treatment with *B. amyloliquefaciens* live cells and SPE-surfactin suppresses grey leaf spot on perennial ryegrass

Our aim in this study was to investigate the possible role of surfactin in eliciting ISR in the perennial ryegrass–*M. oryzae* pathosystem by taking advantage of the $\Delta bmyA\Delta fenA$ mutant *B. amyloliquefaciens* FZB42-AK3 strain (Koumoutsis *et al.*, 2004). *Bacillus amyloliquefaciens* FZB42 is a well-known rhizobacterium with *in vitro* antifungal activity against multiple plant-pathogenic fungi, such as *Fusarium oxysporum* and *Gaeumannomyces graminis* (Grosch *et al.*, 2001; Koumoutsis *et al.*, 2004). Furthermore, the fungal disease suppression ability of this strain has been observed in multiple plant pathosystems, including tomato, cucumber, cotton and, most recently, lettuce (Chowdhury *et al.*, 2013). However, to our knowledge, this is the first report showing that live cells of *B. amyloliquefaciens* and SPE-enriched surfactin are able to suppress grey leaf spot disease in perennial ryegrass.

Comparable suppression of grey leaf spot disease through root application of live cells of wt *B. amyloliquefaciens* FZB42 and its mutant AK3 ($\Delta bmyA::Em^r$, $\Delta fenA::Cm^r$) indicates that bacillomycin D and fengycin are dispensable for ISR activity in perennial ryegrass (Fig. 1a). Furthermore, 250 µg/mL (241.3 µM) SPE-surfactin was sufficient for ISR-mediated suppression of *M. oryzae* (Fig. 1b), with no inhibitory effect on fungal spore germination and appressorium formation (Fig. S2, see Supporting Information). Another CLP, massetolide A (100 µg/mL), from *Pseudomonas fluorescence* SS101, was found to suppress late blight severity on tomato leaves (Tran *et al.*, 2007), suggesting that different plant pathosystems could vary considerably in resistance activity depending on the application rate of specific bacterial determinants. Fourier transform-mass spectrometric fragmentation pattern analyses of different surfactin homologues from AK3 and standard surfactin (Sigma-Aldrich, St. Louis, MO, USA) were an exact match, suggesting that the amino acid sequences of the heptapeptide ring of surfactin isomers were identical (ELLDVLL) (Fig. S3, see Supporting Information). AK3 strain-generated surfactin exhibited a relative proportion of C12 : C13 : C14 : C15 = 2.7:42:49 (%) (Fig. S4, see Supporting Information), which is in agreement with the requirement of C13 or longer acyl chain-bearing surfactin homologues for the generation of any ISR activity (Henry *et al.*, 2011).

The FZB42 strain has been observed to efficiently colonize root surfaces of *Arabidopsis*, corn (Fan *et al.*, 2012), rice (Wang *et al.*, 2013), lettuce (Chowdhury *et al.*, 2013) and wheat (Talboys *et al.*, 2014). Recovery of live FZB42 and AK3 cells from the perennial

ryegrass rhizosphere indicated that they also efficiently colonize the root zone of this grass species. Furthermore, symptomatic leaf blades of mock-treated control plants exhibited predominantly lesion type IV, whereas treated plant infected leaves shared almost equal proportions of lesion types II, III and IV (Fig. 1d). The appearance of different types of lesions suggests that systemic resistance induced by AK3 cells and SPE-surfactin resembles the resistance phenotype of quantitative trait loci-governed partial resistance (Curley *et al.*, 2005; De Vleeschauwer *et al.*, 2006).

H₂O₂-mediated defence responses in perennial ryegrass following AK3 live cell and SPE-surfactin treatment against *M. oryzae*

Similar to surfactin-induced H₂O₂ generation in tobacco cell culture, and in tomato and tobacco root tissues (Henry *et al.*, 2011; Jourdan *et al.*, 2009), SPE-surfactin application resulted in enhanced H₂O₂ accumulation in perennial ryegrass root tissues (Fig. S5, see Supporting Information). Based on the DAB-positive cellular phenotypes observed, treatment with AK3 cells and SPE-surfactin resulted in an enhanced attacker-induced ROS (H₂O₂) production that was characterized by a timely oxidative burst and often encompassed one or more underlying layers of mesophyll cells at the attempted penetration sites by the fungal appressoria (Fig. 2a,b). Among all the DAB-positive interaction types, type II reaction was present in the treated plants at both 36 and 48 hpi (Fig. 2b), but only weakly present at 36 hpi in mock-treated control plants (Figs 2b and S1b). Comparable strong deposition of DAB-positive H₂O₂ inside CWAs in barley (*Hordium vulgare*) leaves thwarted *Blumeria graminis* f. sp. *hordei* (Bgh) penetration attempts (Thordal-Christensen *et al.*, 1997). Similarly, in the *WRKY45*-overexpressing rice–*M. oryzae* interaction, H₂O₂ accumulated in the cell wall, between the cell wall and plasma membrane, immediately underneath the fungal appressorium and around the penetration peg, thereby blocking fungal invasion (Shimono *et al.*, 2012). This phenotypic similarity prompted us to investigate the peroxidase activity in the different cellular fractions of infected leaves. Indeed, a rapid enhancement of peroxidase activity in the cell wall and intercellular fluid (ICF) fractions of the treated plants was observed (Fig. S6, see Supporting Information). However, DAB type III reaction closely resembled a DAB-negative reaction type observed by De Vleeschauwer *et al.* (2008) in the pseudobactin (from *Pseudomonas fluorescens* WCS374r rhizobacterium)-treated rice–*M. oryzae* interaction. In addition, IH were observed to proliferate and spread to secondary cells which, together with the lack of cytoplasmic granulation or browning of the whole cell (Fig. 2a, III), indicated that type III reaction was a sensitive/compatible-type interaction. However, the presence of DAB type IV–VI reactions revealed that a proportion of appressoria triggered additional layers of defence response, including the HR-type reaction, following successful penetration

(Fig. 2a, IV–VI). A comparative analysis of DAB reaction types at two different time periods suggested that application of AK3 live cells and SPE-surfactin generated higher frequencies of enhanced and pathogen-blocking H₂O₂-positive CWAs (type II), as well as whole-cell HR-type incompatible interactions (types IV–VI), relative to that in mock-treated control plants (Fig. 2b).

H₂O₂-dependent, peroxidase-mediated cross-linking of cell wall-localized phenolic compounds plays an important role in strengthening plant cell walls and limiting pathogen invasion (Almagro *et al.*, 2009). Robust accumulation of fluorescent phenolic compounds inside CWAs, and in multiple cells surrounding the attempted penetration sites (Fig. 3b,c), suggested a rapid onset of cell wall fortification in AK3- and SPE-surfactin-treated plants. Similar deposition of phenolic compounds in the barley–*Bgh* (Huckelhoven, 2007) and rice–*M. oryzae* (Faivre-Rampant *et al.*, 2008) interactions was directly proportional to fungal penetration failure. In addition, callose (β-1,3-glucan) deposition is recognized to reinforce weakened or compromised sections of the plant cell wall (Daudi *et al.*, 2012). Augmented deposition of callose was observed in treated plants, together with significantly more non-penetrated callose spots, relative to mock-treated controls (Fig. 5 and Table 2). Rhizobacteria-mediated activation of ISR, involving cell wall reinforcement and accumulation of H₂O₂ and defence-related phenolics, following pathogen challenge, has been described (Ahn *et al.*, 2007; De Vleeschauwer *et al.*, 2008). In *Arabidopsis*, *Prx33* and *Prx34* knockdown lines (*AtPrx33–34 kd*), which are deficient in generating cell wall/apoplastic H₂O₂ following microbe-associated molecular pattern (MAMP) (Flg22) treatment, exhibited a significantly reduced level of callose deposition (Daudi *et al.*, 2012). These *AtPrx33–34 kd* lines also exhibited larger leaves than wt, indicating their inverse relationship with leaf expansion (Daudi *et al.*, 2012). Noticeably, drought-induced ionically bound cell wall peroxidases showed a similar relationship to leaf expansion in annual ryegrass (*Lolium temulentum* L.) (Bacon *et al.*, 1997). In this study, we also observed pathogen-mediated induction of comparable ionically bound cell wall peroxidase activity in AK3- and SPE-surfactin-treated perennial ryegrass (Fig. S6). Depending on the physiological conditions, peroxidases may act as either H₂O₂ scavengers or generators (Almagro *et al.*, 2009; O'Brien *et al.*, 2012). Hence, our results suggest that peroxidase activity was up-regulated in AK3- and SPE-surfactin-treated perennial ryegrass following *M. oryzae* inoculation, which resulted in the enhanced generation of H₂O₂, thereby activating a cascade of events leading to the augmented deposition of callose and phenolic cross-linking of lignin precursors. However, progression of IH in leaf cells of treated plants was quite limited over the 72-hpi time period relative to that in mock-treated control plants (Fig. 6a–h). Similar restricted intracellular proliferation of *M. oryzae* was observed in resistant (ABR5) *Brachypodium distachyon* leaf epidermal cells (Parker *et al.*, 2009).

Enhanced expression of defence-related genes is associated with the protection of perennial ryegrass against *M. oryzae*

ISR-facilitated induction of defence-related *PR* genes following pathogen challenge has been identified in several plant pathosystems (Ahn *et al.*, 2007; Niu *et al.*, 2011), including *Harpophora oryzae*-primed defence genes in the rice–*M. oryzae* interaction (Su *et al.*, 2013) and *B. amyloliquefaciens* CM-2-induced *PR* genes in the tomato–*Ralstonia solanacearum* interaction (Tan *et al.*, 2013). Similarly, comparative analyses of the relative expression levels of all defence-related genes at 0 hpi and later time points suggested a possible 'priming' (Pieterse *et al.*, 2014) effect of AK3 live cell and SPE-surfactin treatments (Fig. 7a–f).

LpPrx (Eaton *et al.*, 2010), a homologue of barley *Prx7* class III peroxidase, which exhibits targeted localization in the CWAs following inoculation with *Bgh* (An *et al.*, 2006; Kristensen *et al.*, 1999), displayed a rapid accumulation in AK3- and SPE-surfactin-treated plants (Fig. 7a). Notably, the relative expression pattern of *LpPrx* complemented the temporal distribution of cell wall and ICF peroxidase activity. Another well-known candidate for pathogen-induced H₂O₂ generation in plant cells is mesophyll-localized germin-like OXO (Almagro *et al.*, 2009). *OXO4*, a perennial ryegrass wound-, H₂O₂- and methyljasmonate (MeJA)-inducible OXO (Le Deunff *et al.*, 2004), exhibited a stronger magnitude of expression in treated plants relative to mock-treated control plants (Fig. 7b), suggesting that *OXO4* generated delayed accumulation of H₂O₂ in leaf mesophyll tissue cell wall, resulting in HR-type PCD (Fig. 2a, IV).

Although the timing of the onset of both *PAL* and *LOXa* up-regulation was similar in treated and mock-treated control plants following *M. oryzae* inoculation, the magnitude of expression, by 24 hpi, was much higher in the former than in the latter (Fig. 7c,d). Rapid up-regulation of these transcripts in treated plants suggested the accumulation of defence-related phenolic metabolites, generated through the activation of phenylpropanoid and lipoxygenase pathways. Enriched activity of both *PAL* and *LOX*, and only *LOX*, was observed in tobacco cell culture and tomato plants, respectively, following surfactin application (Jourdan *et al.*, 2009; Ongena *et al.*, 2007).

We have demonstrated previously that the SAR activator benzothiadiazole (BTH) suppresses grey leaf spot in perennial ryegrass with enhanced expression of *PR-1*, *PR-3.1* and *PR-5* (Rahman *et al.*, 2014). However, no significant differential expression of any of these *PR* genes was detected in the present study. However, enhanced expression of two putative defensin genes, *LpTHb* and *LpDEFa*, which were found to be associated with partial resistance against multiple fungal diseases in perennial ryegrass (Dracatos *et al.*, 2008), was observed in our study. In *Arabidopsis* NAC transcription factor *ATAF1* knocked-down lines,

suppression of defensin genes *PDF1.1* and *PDF1.2* was associated with an increased penetration rate by *Bgh*, although deposition of callose at the attempted penetration sites remained unaffected (Jensen *et al.*, 2008). Induced expression of *OXO4*, *LOXa* and putative defensins (*LpTHb* and *LpDEFa*) suggested that AK3 and SPE-surfactin treatment might have potentiated the JA-mediated defence response(s) in perennial ryegrass to impede *M. oryzae* infection. On the contrary, we have found previously that exogenous JA application increases the susceptibility of perennial ryegrass to *M. oryzae* infection (Rahman *et al.*, 2014). However, the final nature of the plant defence responses to be manifested perhaps depends on the synergistic effect of SA- and JA-mediated pathways, as co-application of 0.1 mM JA and 0.1 mM SA in the apoplast resulted in higher accumulation of H₂O₂ and HR-like PCD compared with lone application of each hormone in *Arabidopsis* (Mur *et al.*, 2006). Based on multiple gene expression analyses, we speculate that physiological concentrations of both SA and JA activated (if any) by *M. oryzae* attack in treated perennial ryegrass were sufficient to exhibit a coordinated defence response tailored towards the restriction of fungal growth in infected plant cells. However, our experiments to determine the endogenous SA concentration showed inconclusive results to identify any differential expression of free SA between treated and mock-treated control plants (data not shown). Further studies involving microarray- and metabolomics-based analyses might help to comprehensively identify the signalling pathways associated with AK3- and SPE-surfactin-mediated ISR in perennial ryegrass.

CONCLUSIONS

Pronounced induction of multilayered defence responses, including the rapid accumulation of H₂O₂, phenolic/polyphenolic compounds and callose at the sites of attempted pathogen entry, together with the timely oxidative burst driving single or multicellular HR-type PCD, following *M. oryzae* challenge in treated plants, supports the ISR effect of AK3 cells and SPE-surfactin treatment in perennial ryegrass. Whether or not AK3-induced ISR is strictly dependent on *in situ* surfactin biosynthesis could not be verified as surfactin-deficient mutants of *B. amyloliquefaciens* FZB42 strains were observed by Raaijmakers *et al.* (2010) to exhibit an extremely poor growth rate and lacked motility. Evidently, the role of surfactin is indispensable for ISR activation, root colonization, biofilm formation and as a signalling molecule during extracellular matrix formation by *B. subtilis* (Pérez-García *et al.*, 2011). It is therefore conceivable that the release of surfactin by *B. amyloliquefaciens* FZB42-AK3 into the rhizosphere of perennial ryegrass played a vital role in the ISR-mediated suppression of *M. oryzae* infection. Surfactin molecules predominantly bind to the plant cell membrane, and such perception of surfactin molecules by plant cells has been primarily attributed to its ISR activity (Henry *et al.*, 2011). Presumably, such

perception could be responsible for the sensitization of the resistance activity of plants, resulting in a surveillance state that is extremely sensitive to *M. oryzae* penetration attempts, thereby triggering a rigorous activation of H₂O₂-mediated defence responses. However, *M. oryzae*-induced modification of host cell metabolic profiles (increased anti-oxidant molecules) (Parker *et al.*, 2009), as well as the requirement of peroxidase and laccase activity by pathogenic *M. oryzae* to scavenge/neutralize host cell-accumulated H₂O₂ activity (Chi *et al.*, 2009), amply supports the importance of H₂O₂-induced defence responses in suppressing *M. oryzae* proliferation. The most recent demonstration of the *M. oryzae* redox potential to surmount the H₂O₂-mediated oxidation potential in non-induced susceptible plants (Samalova *et al.*, 2014) supports our results indicating that prompt and robust induction of H₂O₂-mediated multifaceted defence responses in AK3- and SPE-surfactin-treated perennial ryegrass plays a major role in grey leaf spot disease suppression.

EXPERIMENTAL PROCEDURES

Plant materials and microbial culture conditions

All experiments were conducted in controlled-environment chambers with perennial ryegrass (*Lolium perenne* L.) cv. Legacy-II, a highly susceptible cultivar to infection by *M. oryzae*, grown in plastic pots (diameter, 10.2 cm) with a seeding rate of 20 g/m² in peat Farfard mix no. 2 : sand (2:1, v/v), as described previously (Rahman *et al.*, 2014). Rhizobacterium *B. amyloliquefaciens* FZB42 and its mutant strain AK3 ($\Delta bmyA::Em^r$, $\Delta fenA::Cm^r$) were collected from the Bacillus Genetic Stock Center (BGSC, Columbus, OH, USA) (Dean and Zeigler, 1994) and were maintained as recommended by BGSC. The pathogenic isolate of *M. oryzae* OST was routinely grown on potato dextrose agar (PDA) at 25 °C. The preparation of *M. oryzae* conidia was identical to that described previously (Rahman *et al.*, 2014).

Magnaporthe oryzae inoculation and disease rating

Four-week-old perennial ryegrass plants were inoculated with (3–4) × 10⁴ spores/mL conidial suspension by atomizing the leaves until run-off, as described previously (Rahman *et al.*, 2014). Pots of plants were arranged in a randomized complete block design with three replications for each treatment. DI and DS were assessed 5 days after inoculation with *M. oryzae*, as described previously (Rahman *et al.*, 2014). In brief, DI was assessed by quantifying the percentage of symptomatic leaves in each treatment. DS was assessed using an index of 0–10 (0, no necrosis; 10, >90% necrotic leaf area on individual blades). The rating scale used for different lesion types on the symptomatic leaf blades was measured according to the severity of the lesions described in Table 1 (Curley *et al.*, 2005). Between 100 and 150 leaf blades from each pot of plants were analysed. DI and DS experiments were repeated twice and disease lesion type (DLT) experiments were repeated once. All lesion type data generated from control and treated plants in each experiment were used for significance analysis independently. Data from each experiment were subjected

to analysis of variance (ANOVA) using MINITAB (version 16.1.0; Minitab Inc., State College, PA, USA). Multiple comparisons of means for DI and DS, and for each lesion type (for DLTs), were made using Student–Newman–Keul's (SNK) test ($P < 0.05$).

Bacterial inocula preparation and surfactin extraction

Bacillus amyloliquefaciens strains were prepared as described previously (Ongena *et al.*, 2005). In brief, bacterial cells were grown at 30 °C for 72 h and 180 rpm in 'Opt medium' (Jourdan *et al.*, 2009). Cultures were then centrifuged at 15 000 *g* for 15 min, washed twice with sterile saline water (0.85% NaCl) and re-suspended in sterile saline water to make 5×10^7 colony-forming units (CFU)/mL. An enriched surfactin lipopeptide extract was prepared using the SPE method described previously (Ongena *et al.*, 2005) from a culture supernatant of the AK3 strain grown in 'Opt medium' (Jourdan *et al.*, 2009).

ISR experiments

ISR experiments were performed using both live AK3 cells and SPE-surfactin, as described previously (De Vleeschauwer *et al.*, 2008). Briefly, 'Legacy-II' perennial ryegrass (*L. perenne* L.) seeds were first surface sterilized with 1% sodium hypochlorite and air dried. Seeds were then soaked in AK3 live cell suspension in sterile saline (5×10^7 CFU/mL) for 30 min. In addition, the bacterial inoculum was thoroughly mixed with autoclaved potting mix (pot diameter, 10.2 cm) to a final density of 5×10^7 CFU/g and again after 3 weeks as a root drench. Mock-treated control plants were treated with equal volumes of 0.85% sterile saline water. A 10-mL solution of SPE-surfactin (250 µg/mL) was applied once as a soil drench 2 days prior to pathogen challenge, unless otherwise stated.

Evaluation of bacterial colonization of plant roots

Plant root colonization and possible migration of root-inoculated bacterial cells to distal leaf tissues were determined according to Kinsella *et al.* (2009) with minor modifications (Ongena *et al.*, 2005). Briefly, after 5 weeks of growth, 1-g samples of leaf and shoot tissue or root fresh weight (FW) were randomly collected from three independent pots of plants per experiment. Root samples were lightly washed with sterile Millipore water to remove clinging potting medium. Both tissue samples were then homogenized in 20 mL of sterile Millipore (Darmstadt, Germany) water with 0.05% Tween 20 and incubated for 1 h at 140 rpm. Following incubation, aliquots of 100 µL of serial dilutions were plated on 'Turner and Backman selective medium' (TBSM) (Kinsella *et al.*, 2009) and incubated for 3 days at 24 °C. Furthermore, cyclohexamide (45 µg/mL) was added to inhibit fungi and polymyxin B (22.5 µg/mL) to inhibit Gram-negative bacteria. When required, chloramphenicol (5 µg/mL) and erythromycin (1 µg/mL) were added for AK3 selection. FZB42 colonies on the TBSM plates had a characteristic morphology, which was easily distinguishable, and formed the large majority of the total CFUs.

Visualization of defence responses

The production of H₂O₂ and reactive oxygen intermediates (ROIs) was detected using the 'DAB-uptake method' (Thordal-Christensen *et al.*,

1997) with modifications (Daudi *et al.*, 2012). Pieces of leaves (3 cm) were infiltrated with 1 mg/mL DAB containing Tween 20 (0.05% v/v) and 10 mM sodium phosphate buffer (pH 7.0) for 5 h. Leaves were then fixed in ethanol : glycerol : acetic acid (3:1:1, v/v) until clear, mounted in 60% glycerol (v/v) and visualized by bright-field (BF) microscopy. Phenolic compounds were analysed as autofluorescence in cleared leaf segments and visualized under blue light epifluorescence (Nikon Eclipse E600, Melville, NY, USA; GFP filter set 480/30 nm; DM 400 dichroic beam splitter 505 nm and emission filter 535/40 nm) or by their blue/turquoise appearance when stained in a solution of 0.05% toluidine blue in 50 mM citrate buffer, pH 3.5, visualized by BF microscopy (Mellersh *et al.*, 2002). For the visualization of callose deposition at the infection sites, an identical technique with 0.05% aniline blue stain was employed as described previously (Rahman *et al.*, 2014). IH were visualized using a modified KOH–aniline blue technique (Hood and Shew, 1996). In brief, cleared leaves were boiled for 10 min in 1 M KOH, followed by staining with 0.05% aniline blue, and visualized using BF microscopy with a UV filter (Olympus BX-61, Melville, NY, USA; excitation filter 350/50 nm, replicated DM 400 dichroic beam splitter and emission filter 460/50 nm).

In each experiment, 75–125 single-cell interaction sites on leaf segments (length, 3 cm), randomly collected from the middle part of inoculated leaves from all experimental pots (replicates) and pooled together, were inspected and scored independently. All experiments were repeated twice. All BF and UV-filtered images were acquired digitally [Hamamatsu cooled digital camera: ORCA ER (Model C4742-80) (Bridgewater, NJ, USA) and Olympus DP71 (Center Valley, PA, USA)] and further processed with CellSens Dimension software.

Analysis of gene expression using quantitative real-time PCR

The collection of leaf samples, RNA extraction, cDNA synthesis and quantitative RT-PCR were identical to those described previously (Rahman *et al.*, 2014). Specific primers for the perennial ryegrass genes *LpOXO*, *LpPxx*, *LpPALa*, *LpLOXa*, *LpTHb*, *LpDEFa* and *LpeEF1A(s)* (stably expressed eukaryotic elongation factor 1 α) were used for each cDNA sample (Table 3). For each gene, the sample value at the 0-h time point of healthy control plants was defined as the calibrator sample and the results were expressed as the fold change of mRNA over this sample using the comparative threshold method ($2^{-\Delta\Delta C_T}$) (Livak and Schmittgen, 2001). Primers for each gene were chosen on the basis of the amplification efficiency between a target and the reference gene [*eEF1A(s)*], which was determined as described previously (Livak and Schmittgen, 2001). The specification of each pair of primers was also confirmed by randomly sequencing PCR products and further consolidated by melting curve analysis using real-time PCR. All experiments were performed three times and each real-time PCR sample was run in triplicate.

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Table 3 DNA primers used to assay the gene expression by quantitative real-time PCR.

Gene	Primer	Primer sequence (5'–3')	Product size (base pair)	Reference
<i>Prx</i>	Forward	CCGGAACACACCAGAATGAA	111	Eaton <i>et al.</i> (2010)
	Reverse	TATGACGTACGGGTTCGATGA		
<i>OXO4</i>	Forward	CGACTCTGGGAACAAGCTATAC	139	Le Deunff <i>et al.</i> (2004)
	Reverse	CGACTCTGGGAACAAGCTATAC		
<i>PAL</i>	Forward	GCTTGAAGGGTGCTGAGATT	131	Sawbridge <i>et al.</i> (2003)
	Reverse	AGAGATGAGACCAGAGAGTTG		
<i>LOX</i>	Forward	TCGCTGAATCGCTTGAACA	136	Eaton <i>et al.</i> (2010)
	Reverse	TCCAGACCATCATCGGGATA		
<i>THb</i>	Forward	GTGAGCTCGATCGGTTAGTG	74	Dracatos <i>et al.</i> (2008)
	Reverse	AGAAAGTGCCATCTCTGCTTG		
<i>DEFa</i>	Forward	CTGTGTTGCTAATCTTGCGC	84	Dracatos <i>et al.</i> (2008)
	Reverse	CAGCACTTAATTTTCAGGGTCTAC		
<i>eEF1A(s)</i>	Forward	CCGTTTTGTCGAGTTGGT	113	Lee <i>et al.</i> (2010)
	Reverse	AGCAACTGTAACCGAACATAGC		

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Representative images of perennial ryegrass leaf blades treated with 3,3'-diaminobenzidine (DAB), 24 and 36 h following inoculation with *Magnaporthe oryzae*. Ap, appressorium.

Fig. S2 Effect of solid-phase extraction (SPE)-enriched cyclic lipopeptide(s) from wild-type (wt) *Bacillus amyloliquefaciens* FZB42 and AK3 strains on germination of ZsGreen-tagged *Magnaporthe oryzae*. Ap, appressorium.

Fig. S3 Reverse phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (MS) analysis of surfactin isomers in solid-phase extraction-enriched surfactin (SPE-surfactin) (a1, a2 and a3) and standard surfactin (b1, b2 and b3) (Sigma-Aldrich).

Fig. S4 Relative proportions of different surfactin homologues in standard surfactin (Sigma-Aldrich) (a) and solid-phase extraction-enriched surfactin (SPE-surfactin) (b).

Fig. S5 Hydrogen peroxide (H₂O₂) content in perennial ryegrass roots following application of 250 µg/mL of solid-phase extraction-enriched surfactin (SPE-surfactin).

Fig. S6 Time course of peroxidase enzyme activity in different cellular fractions of perennial ryegrass leaf cells following infection with *Magnaporthe oryzae*: (a) cell wall (CW); (b) intercellular fluid (ICF); (c) cytoplasm.