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Role of cyclic lipopeptides produced by *Bacillus subtilis* in mounting induced immunity in rice (*Oryza sativa* L.)

Sabrina Chandler [a], Nathalie Van Hese [a], François Coutte [b], Philippe Jacques [b], Monica Höfte [a,*], David De Vleesschauwer [a]

[a] Laboratory of Phytopathology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium
[b] Charles Viollette Institute, Regional Research Laboratory for Food and Biotechnology – ProBioGEM Team, EA1026, Polytech'Lille, University Lille 1, Sciences and Technologies, Avenue Paul Langevin, F-59 655 Villeneuve d'Asq, France

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ABSTRACT

In this study, we demonstrate the potential of *Bacillus subtilis* BBG111 to trigger ISR in rice (*Oryza sativa* L.) against *Rhizoctonia solani*, while there was no effect against *Magnaporthe oryzae*. We show that plant recognition of BBG111 induces jasmonic acid (JA) and ethylene (ET) as well as abscisic acid (ABA) and auxin signaling. In addition, *B. subtilis* supernatants also boosted immune responses triggered by chitin, suggesting that BBG111 triggers ISR at least in part by reinforcing basal plant defense responses. Finally, our results reveal an indispensable role of the BBG111 cyclic lipopeptides, fengycin and surfactin, in the induced defense state.

1. Introduction

Rice (*Oryza sativa* L.) is the most consumed staple food grain for more than three billion people living in tropical and subtropical Asia [1]. However, rice production is severely affected by a variety of biotic and abiotic factors including pests, weeds, drought, heat and salinity. In addition, more than 70 diseases caused by bacteria, fungi, viruses or nematodes have been recorded on rice, among which rice blast (*Magnaporthe oryzae*) and sheath blight (*Rhizoctonia solani*) are the most important fungal constraints on high productivity [2,3].

The filamentous ascomycete *M. oryzae* (Hebert) Barr [anamorph *Pyricularia oryzae* Cavara syn. *Pyricularia grisea*] is one of the most devastating pathogens of rice worldwide due to its widespread distribution and destructiveness [4]. A recent survey saw *M. oryzae* ranked as the world's top 1 plant-pathogenic fungus based on scientific/economic importance [5]. *M. oryzae* is a hemibiotrophic pathogen as its infection cycle combines successive biotrophic and necrotrophic growth stages. Following appressorium-mediated penetration, successful infection requires an initial period of biotrophy during which the fungus forms bulbous invading hyphae within apparently healthy plant cells [6]. Once established within the plant, the pathogen gradually switches to a necrotrophic lifestyle, resulting in the appearance of visual disease symptoms. *R. solani* Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk], on the other hand, is an archetypal necrotroph which kills host cells at early stages of the infection process and feeds on the remains.

Management of fungal rice diseases usually involves the use of fungicides and cultivation of resistant varieties. However, whilst

* Corresponding author. Department of Crop Protection, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.
E-mail address: monica.hofte@ugent.be (M. Höfte).

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the use of hazardous chemicals is environmentally undesirable as well as economically costly, resistant blast cultivars often do not withstand more than one or two years of cultivation before succumbing to diseases, due to either breakdown or gradual erosion of the resistance in face of the high variability of the pathogen population [7]. Moreover, though partial resistance to sheath blight has been reported, no major resistance genes have been identified despite screening more than 3000 accessions of germplasm worldwide [8]. Hence, there is considerable incentive to develop new disease control strategies providing durable, environmentally sound, and broad-spectrum pathogen protection. Among such strategies, approaches capitalizing on the plant's own defensive repertoire seem especially promising [9].

To effectively combat invasion by microbial pathogens, plants have evolved sophisticated mechanisms providing several strategic layers of coordinated defenses. Pre-formed structural and physical barriers as well as inducible plant defenses that are activated upon pathogen recognition constitute the first line of defense and result in a basal level of immunity [10]. Besides these primary attacker-specific responses, plants can also mount a non-specific systemic resistance response that is effective against future pathogen attack. Depending on the organism interacting with the plant, plants are able to activate several types of this so-called induced resistance, including systemic acquired resistance (SAR). SAR is triggered by a localized infection with a necrotizing pathogen and is marked by local and systemic increases in salicylic acid (SA) and the accumulation of pathogenesis related (PRs) proteins [11]. Colonization of the roots by selected strains of plant growth-promoting rhizobacteria (PGPR) leads to a phenotypically similar form of induced resistance, commonly referred to as induced systemic resistance (ISR) [12]. Seminal studies in *Arabidopsis* and rice have shown that rhizobacteria-mediated ISR often functions independently of SA but requires components of the jasmonic acid (JA) and ethylene (ET) response pathways [13–15]. However, much progress has since been made and is now becoming increasingly clear that various hormone-dependent signaling conduits can govern the ISR phenotype depending on the rhizobacterium and the plant–pathogen system used [16].

Over the past few years, multiple PGPR strains have been shown to successfully control rice blast and sheath blight diseases. These include various *Pseudomonas* and *Bacillus subtilis* strains, including *Pseudomonas fluorescens* PF1 and FP7 [17], *P. fluorescens* PfALR2 [18], and *B. subtilis* MBI 600 [19], all of which are effective against sheath blight, as well as *Pseudomonas aeruginosa* 7NSK2 and *P. fluorescens* WCS374r, which are both effective against rice blast [15,20]. However, the ability of the strains *B. subtilis* BBG111 and RFB104 to trigger ISR in rice has not been tested yet.

B. subtilis strains BBG111 and RFB104 are two strains that are known to produce different pattern of cyclic lipopeptides (CLP). Composed of a cyclized oligopeptide lactone or lactam ring coupled to a fatty acid [21,22], CLPs are known for their powerful activity against a wide range of organisms, including fungi, bacteria, protozoa and plants and they also have low environmental toxicity [23–26]. *B. subtilis* strain BBG111 produces two CLPs, fengycin and surfactin [27], whereas *B. subtilis* RFB104, a derivate of *B. subtilis* ATCC6633, produces surfactin and mycosubtilin [28]. Ongena et al. [29] have already shown the importance of surfactin and fengycin in induced systemic resistance in bean, but no studies thus far have evaluated their impact on ISR in cereal crops.

In the present study, we demonstrate the ability of *B. subtilis* BBG111 to mount ISR against sheath blight, while no effect on rice blast was noticed. Furthermore, we show the involvement of CLPs in the establishment and maintenance of ISR and study their mode of action by analyzing defense gene expression, hormone pathway activation and cell death responses.

2. Materials and methods

2.1. Plant materials and cell cultures

Seeds of rice (*Oryza sativa* subsp. *japonica*.) cultivar Taichung 65 (abbreviated as T65), were used throughout this study. Unless stated otherwise, rice plants were grown in commercial potting soil (Structural; Snebbout) under growth chamber conditions (constant temperature of 28 °C, relative humidity: 60%, 12/12 light/dark period). Cell suspension cultures of rice cultivar Kitaake (*Oryza sativa* subsp. *japonica*) were grown in the dark on a rotary shaker (120 rpm) at 28 °C in liquid AA medium [30]. The cells were diluted in fresh medium every week, and all experiments were performed 5 days after transfer.

2.2. Cultivation of rhizobacteria and pathogens

Bacterial strains used in this study are listed in Table 1. *B. subtilis* strains BBG111 and RFB104 were routinely grown for 24–28 h at 28 °C on Luria–Bertani (LB) agar plates. Alternatively, a single bacterial colony was inoculated in LB broth and grown for 24–28 h at 28 °C under shaking conditions (150 rpm). Bacterial suspensions were adjusted to the desired concentration based on their optical density at 620 nm.

M. oryzae isolate VT5M1 [31] was grown at 28 °C on potato dextrose agar (PDA) in darkness. Seven-day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light (combination of Philips TLD 18W/08 and Philips TLD 18W/33) for 7 days to induce sporulation. Conidia were harvested according to De Vleesschauwer et al. [20], and inoculum concentration was adjusted to a final density of 5×10^4 spores mL^{-1} in 0.5% gelatin (type B from bovine skin; Sigma–Aldrich G-6650).

R. solani isolate MAN-86 (AG-1, IA) [32], obtained from symptomatic plants (cv. IR-50) in rice fields in the state of Karnataka (India) was maintained on Potato Dextrose Agar (PDA) medium at 28 °C in the dark.

2.3. ISR bioassays

Induced resistance bioassays were performed essentially as described by De Vleesschauwer et al. [20]. Briefly, plants were grown under growth chamber conditions (28 °C, relative humidity: 60%, 12/12 light regimen) in commercial potting soil (Structural; Snebbout) that had been autoclaved twice on alternate days for 21 min. Rice seeds first were surface sterilized with 1% sodium hypochlorite for 2 min, rinsed three times with sterile, demineralized water, and incubated on wet sterile filter paper for 5 days in the dark at 28 °C to germinate. The bacterial inoculum was thoroughly mixed with the potting soil to a final density of 5×10^7 cfu g^{-1} and, 12 days later, applied a second time as a soil drench (5×10^7 cfu g^{-1}). In control treatments, soil and rice plants were treated with equal volumes of sterilized saline solution.

For chemical induction of resistance against *M. oryzae*, plants were treated with benzothiadiazole (BTH) at 3 days prior to challenge inoculation. BTH (BION 50 WG), formulated as a water-dispersible granule containing 50% active ingredients, was dissolved in sterilized demineralized water for use and applied as a soil drench. Control plants were treated with an equal volume of water. BTH was provided by Syngenta Crop Protection.

2.4. Pathogen inoculation and disease rating

Five-week-old rice seedlings (five-leaf stage) were challenge inoculated with *M. oryzae* isolate VT5M1 as described before [20]. Seven days after inoculation, disease was assessed by counting the