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NRPS-Derived Isoquinolines and Lipopeptides Mediate Antagonism between Plant Pathogenic Fungi and Bacteria

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Abstract

Bacterial–fungal interactions are presumed to be mediated chiefly by small-molecule signals; however, little is known about the signaling networks that regulate antagonistic relationships between pathogens. Here, we show that the ralstonins, lipopeptides produced by the plant pathogenic bacteria *Ralstonia solanacearum*, interfere with germination of the plant-pathogenic fungus *Aspergillus flavus* by down-regulating expression of a cryptic biosynthetic gene cluster (BGC), named *imq*. Comparative metabolomic analysis of overexpression strains of the transcription factor ImqK revealed *imq*-dependent production of a family of tripeptide-derived alkaloids, the imizoquins. These alkaloids are produced *via* a nonribosomal peptide synthetase- (NRPS-)derived tripeptide and contain an unprecedented tricyclic imidazo[2,1-*a*]isoquinoline ring

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S.K., J.A.B., and J.E.S. performed and executed the experiments. M.I., N.P.K., and F.C.S. provided materials and equipment. S.K., J.A.B., J.E.S., N.P.K., and F.C.S. conceived and designed the study and wrote the manuscript.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchem-bio.7b00731. Description of fungal and bacterial strains and culture conditions, genetic manipulations, comparative metabolomics by LC-MS, analytical methods and equipment, chromatographic enrichment and structural elucidation of imizoquins; Supporting Figures S1–S5 relating to *imq* expression and impact on growth and germination, LC-MS, and biosynthesis analyses; Supporting Figures S6–S10 describing the mechanism and generality of the imizoquins' effect on germination and bacterial growth; Supporting Figure S12 showing southern confirmation of all *A. flavus* mutants; Supporting Tables S1–S5 providing supporting information on RNA seq analysis, HR-LCMS, *imq* homology, and PCR primer sets; Supporting Tables S6–S10 and associated NMR spectra showing ¹H and ¹³C chemical shifts, ¹H–¹H COSY correlations and coupling constants, and ¹H–¹³C HMBC correlations for the identified compounds (PDF)

system. We show that the imizoquins serve a protective role against oxidative stress that is essential for normal *A. flavus* germination. Supplementation of purified imizoquins restored wildtype germination to a *imqK* *A. flavus* strain and protected the fungus from ROS damage. Whereas the bacterial ralstonins retarded *A. flavus* germination and suppressed expression of the *imq* cluster, the fungal imizoquins in turn suppressed growth of *R. solanacearum*. We suggest such reciprocal small-molecule-mediated antagonism is a common feature in microbial encounters affecting pathogenicity and survival of the involved species.

Graphical Abstract

Soil-associated microbial populations represent complex and dynamic microbiomes that communicate primarily through chemical signaling. Several recent studies demonstrated the importance of specialized metabolites derived from biosynthetic gene clusters (BGCs), including nonribosomal peptide synthetase- (NRPS), polyketide synthase- (PKS), and terpene synthase-based clusters, in mediating specific interactions between bacteria and fungi.^{1,2} Expression of most of these BGCs is strongly regulated by specific growth conditions or confrontations with other organisms. In fact, some otherwise silent BGCs are activated during interkingdom encounters,³ whereas others are repressed during such encounters.⁴

Agricultural environments may include plant pathogens otherwise not found in the rhizosphere. In addition to many soil dwelling fungal pathogens, the wilt bacteria *Ralstonia solanacearum* infects a multitude of crops and persists in the soil through unclear mechanisms, potentially by colonizing weeds that remain asymptomatic.⁵ We recently found that *R. solanacearum* produces hybrid PKS-NRPS-derived lipopeptides, the ralstonins (originally called ralsolamycin), that induce chlamydospore formation across disparate taxa of fungi, which facilitates bacterial entry into chlamydospore tissue, which may benefit bacterial survival.⁶ The structures of the ralstonins were recently characterized in detail by Murai *et al.*⁷ It is not clear, however, whether chlamydospore development provides a competitive advantage to the colonized fungus. Although thick walled chlamydospores can weather harsh environmental conditions, bacterial proliferation inside these spores may decrease survival of the fungus. We therefore hypothesized that ralstonin production, while conferring a fitness benefit to *Ralstonia*, may elicit a defensive response from challenged fungi, or, conversely ralstonin may act to suppress mechanisms favoring fungal over bacterial success.

Here, we characterize antagonistic crosstalk mediated by ralstonin and a newly discovered class of fungal isoquinoline alkaloids, the imizoquins, where each compound retards some aspect of the growth dynamics of the other microbe. Imizoquins stimulate germination of *Aspergillus* spores, whereas ralstonin production by *R. solanacearum* impedes spore germination of *A. flavus* and reduces expression of the imizoquin BGC. Conversely, imizoquins have a slight but significant effect on slowing *R. solanacearum* growth. Mechanistically, imizoquins possess ROS quenching properties that yield protective properties to fungal spores and exhibit conserved germination promoting properties across diverse *Aspergillus* spp. We propose that reciprocal antagonistic signaling molecules, as uncovered in this work, are likely representative of interactions in the microbial rhizosphere.

RESULTS AND DISCUSSION

Ralstonin Downregulates Expression of the *imq* Gene Cluster in *A. f. flavus*

Because the ralstonins induce chlamydospore formation in fungi and facilitate bacterial entry into fungal tissues,⁶ we hypothesized that these compounds may affect expression of fungal BGCs involved in defense. We thus compared RNaseq data of *A. flavus* treated with either *R. solanacearum* wild-type (GMI1000) extract or *rmyA* mutant extract, which is deficient in ralstonin (Figure 1a).⁶ Monitoring *A. flavus* gene expression over a 30-h period, we observed differential expression of 65 genes (fold change >2, FDR-corrected *p* value < 0.05), of which 29 encoded uncharacterized proteins, eight encoded various transporters, and three encoded putative secondary metabolite genes (Supporting Table S1).⁸ Two genes downregulated in GMI1000 versus *rmyA* treatment appeared to belong to a cryptic BGC we named *imq* (Figure 1b) and were further confirmed with Northern blot analysis (Supporting Figure S1). The *imq* BGC encodes a canonical three-module NRPS (*imqB*), a phenol 2-monooxygenase (*imqC*), two transporters (*imqD* and *imqJ*), a gibberellin oxidase (*imqE*), the *N*- and *O*-methyltransferases (*imqF* and *imqG*, respectively), an FAD-dependent oxidase (*imqH*), a C6 transcription factor (*imqK*), as well as two small proteins with no predicted function (*imqA* and *imqI*; Figure 1b). The *imq* cluster is closely related to the *wyk* BGC in *A. oryzae*, which is involved in the biosynthesis of a family of tripeptide-derived isoquinoline alkaloids.^{9,10} Notably, *imqC*, *imqF*, and *imqH* are homologues of *fsqG*, *fsqC*, and *fsqB*, respectively, which we previously showed to carry out the three-step transformation of L-tyrosine into tetrahydroisoquinoline en route to the fumisoquins in *A. fumigatus* via a pathway that is analogous to isoquinoline biosynthesis in plants (Supporting Table S2).¹¹

Identification of *imq*-Derived Metabolites

To identify *imq*-derived metabolites, we created an *A. flavus* overexpression (OE) strain for the putative C6 transcription factor ImqK.¹² Replacement of the *imqK* promoter with the constitutively expressed *gdpA* (OE::*imqK*) induced expression of the other cluster genes (Figure 2a) and production of a purple pigment (Supporting Figure S2). Two cluster flanking genes (AFLA_064220 and AFLA_064340) were not regulated by ImqK and thus likely define the boundaries of the cluster (Figure 2a). To identify *imq*-derived metabolites, we employed liquid chromatography–mass spectrometry (LC–MS)-based comparative metabolomics¹¹ of metabolome extracts of OE::*imqK* and WT *A. flavus*. Since the *imq*

cluster is not expressed in WT under laboratory conditions, any compounds strongly upregulated (or additionally detected) in the OE::*imqK* strain represented candidates for possible *imq*-derived metabolites. We detected six components of the OE::*imqK* metabolome that were absent in the WT extracts (compounds **1–6**, Figure 2c, Supporting Figure S3, and Supporting Table S3), two of which strongly absorbed at 420 nm, accounting for the purple pigmentation of OE::*imqK* cultures. Following chromatographic enrichment, a standard suite of 2D NMR-spectroscopic experiments revealed that these *imq*-dependent compounds, named imizoquin A and B (**1** and **2**, respectively, Figure 2b,c), represent tripeptide-derived alkaloids featuring the unprecedented imidazo[2,1-*a*]-isoquinoline ring system (Figure 2b,c; see Supporting Information for structural elucidation details). The molecular formulas of two additional *imq*-dependent compounds differed from that of imizoquin B (**2**) by ± 2.016 , corresponding to two hydrogen atoms (Supporting Table S3), suggesting that these compounds, named imizoquin C and imizoquin D (**3** and **4**, respectively, Figure 2c), represent different oxidation states of the same scaffold, which was confirmed by 2D NMR spectroscopic analysis (Supporting Tables S6–S9). The remaining two *imq*-dependent metabolites were identified as the previously described isoquinoline alkaloids TMC-2A/B¹³ (**5** and **6**, Figure 2C and Supporting Figure S3), which likely represent intermediates in the biosynthesis of the fused tricyclic imidazo[2,1-*a*]isoquinoline ring system of **1–4**.

Additional Metabolic Profiling and a Model for Imizoquin Biogenesis

ImqB is a canonical three-module NRPS, suggesting assembly of the tripeptide backbone of the imizoquins occurs by standard NRPS assembly line style adenylation, thiolation, and condensation of Trp, Tyr, and Leu derived precursors (Figure 3).¹⁴ *imqC*, *imqF*, and *imqG* are close homologues of the tailoring genes responsible for isoquinoline formation in the *A. fumigatus* *fsq* cluster,¹¹ suggesting that isoquinoline formation en route to the imizoquins proceeds as described for the case of *fsq*-derived metabolites, including *N*-methylation (by ImqF) and phenol oxidation (by ImqC), followed by cyclization *via* the FAD-dependent oxidase ImqH (Figure 3, Supporting Figure S4). Importantly, this sequence requires the presence of a free amine in the tyrosine moiety, indicating that isoquinoline formation occurs *prior* to peptide bond formation. This biosynthetic model is further supported by the identification of an additional *imq*-dependent metabolite, the pyrazino[1,2-*b*]isoquinoline **7** (imizoquin E, see Supporting Table S3 for HR-MS and Supporting Table S10 for NMR spectroscopic data), which likely arises from nucleophilic attack of the Trp-derived amino group onto the thioester-attached dipeptide intermediate (Figure 3). In this model, the imidazolidin-4-one ring could form following additional oxidation of the methyl-derived bridgehead carbon by ImqH. Lastly, *O*-methylation (by ImqG) and leucine hydroxylation (by ImqE) complete biosynthesis of the imizoquins.

Imizoquins Are Required for Normal *A. f. flavus* Germination

With the exception of a purple pigmentation observable in the OE::*imqK* mutant, the *imqK* and OE::*imqK* strains showed little differences from wild-type (WT) in vegetative growth (Supporting Figure S2). However, in assessing the strains for any developmental changes, we found a consistent pattern of delayed germination in *imqK* and accelerated germination in OE::*imqK* compared to WT, such that by 9 h postinoculation (hpi) ca. 80% of OE::*imqK*

spores had germinated compared to 40% of WT and only 10% of *imqK* (Figure 4a and Supporting Figure S5).

To test whether these differences in germination rates were a result of differences in imizoquin production, we added purified imizoquins to *imqK* and WT *A. flavus* cultures and measured germination rates. Imizoquin A and B (**1**, **2**) were added to spores at 0.1 mM, 0.2 mM, and 0.4 mM. As shown in Figure 4b–d, at all tested concentrations the imizoquins increased germination of both *imqK* and WT to above that of untreated OE::*imqK* (imizoquin B (**2**) was only tested on *imqK*). Concentrations above 0.4 mM did not increase the germination rate further (Supporting Figure S6). Testing of the structurally related alkaloids, TMC-2A and TMC-2B (**5** and **6**, respectively),¹³ showed similarly accelerated germination (Supporting Figure S7). While imizoquin biosynthesis is required for germination, these compounds are not sequestered in the spore, as evidenced by LC-HRMS metabolite profiling of **1–7** in isolated conidia vs isolated mycelia (Supporting Figure S3). This suggests that imizoquin biosynthesis is activated early in the process of initiation of germination, as shown below.

Imizoquin Induction of Germination Is Conserved in Closely Related Fungal Species

Next, we asked whether the imizoquins affect germination rates in other fungi, in addition to *A. flavus*. Supporting Figure S8 shows that **1** increases the germination rate of all tested *Aspergillus* species, including the closely related *A. oryzae* and the more phylogenetically distant *A. nidulans* and *A. fumigatus*. Additionally, **1** increased the germination rate of *Penicillium expansum* (a related species in the order Eurotiales) but had little impact on germination of *Fusarium oxysporum* (a distant species in the order Hypocreales). As observed with *A. flavus* (Supporting Figure S6), concentrations above 0.4 mM did not increase germination rates further.

Imizoquins Induce Germination through a ROS Dependent Mechanism

Fungal spore germination is affected by many different, interacting parameters, *e.g.*, density, temperature, growth medium composition, *etc.*^{15,16} However, the identification of a specific family of metabolites that accelerate germination may provide interesting opportunities to investigate mechanisms underlying germination. Because the structures of the imizoquins suggest that they may serve a role in fungal redox chemistry, we tested resilience of the OE::*imqK* and *imqK* mutant strains to oxidative stress induced by hydrogen peroxide,¹⁷ *t*-butyl peroxide, and menadione and assessed growth rate, as well as spore survival (for hydrogen peroxide only; Figure 4e,f). In comparison to wild type, we found that the OE::*imqK* strain showed increased resistance to several ROS generating treatments, whereas the *imqK* strain exhibited enhanced ROS sensitivity in both mycelial growth (Figure 4e) and spore survival (Figure 4f) assays. Comparing imizoquin A and B (**1** and **2**, respectively) to a known antioxidant, uric acid, we found that both compounds exhibited significant antioxidant activities (Supporting Figure S9) and, furthermore, protected spores from the germination inhibitory effects of H₂O₂ (Figure 5a and b). The related metabolites, TMC-2A/B (**5,6**), also showed antioxidant properties (Supporting Figure S9). These data suggested that the pro-germination properties of the identified *imq*-dependent compounds are due to ROS quenching. To further examine this hypothesis, we asked if an alternative

antioxidant, glutathione, could promote germination. Similar to the imizoquins, exogenously added glutathione promoted the germination rate of both WT and *imqK* strains in a concentration-dependent manner (Figure 5c,d).

To assess whether the *imq* cluster is activated early in germination and to test whether ROS is involved in activation of the *imq* cluster, we measured expression of *imq* genes before, during, and post germination by semiquantitative RT-PCR, with and without the addition of a ROS source in WT *A. flavus* (Supporting Figure S10). We found that *imq* cluster expression is activated during early germination but does not appear to be affected by the addition of ROS. Taken together, our data indicate that imizoquins produced early in the germination process underlie the observed ROS protection, and that the upregulation of the *imq* cluster in *A. flavus* upon contact with *R. solanacearum* is triggered by the ralstonins and not ROS.

Impacts of Imizoquins on *Ralstonia* Replication and Ralstonin on *Aspergillus* Germination

On the basis of the results of our transcriptomic analysis, which showed that *imq* cluster expression is downregulated in response to GMI1000 relative to *rmyA* extracts, we hypothesized that the ralstonins, by suppressing *imq* cluster expression, negatively affect fungal germination. To assess potential effects of the ralstonins, we examined if ralstonin-containing GMI1000 extracts affected spore germination. GMI1000 extracts initially delayed germination of all three strains of *A. flavus* compared to the methanol control (Figure 6, note that incorporation of methanol in the extracts delayed even germination of the control strain to 11 hpi). However, germination rates of both the WT and OE::*imqK* mutant exceeded that of methanol control after 14 h in the case of GMI1000 extract, whereas germination rates of the imizoquin-deficient *imqK* strain remained lower than control (Figure 6a–c). In contrast, extracts from the ralstonin-deficient *rmyA* strain promoted germination of all three strains of *A. flavus* in comparison to both the methanol control and GMI1000 extracts (Figure 6a–c). These results suggest an inhibitory effect of ralstonin on spore germination that is counteracted in part by induction of the imizoquin pathway.

Next, we asked whether, conversely, the imizoquins might adversely affect *R. solanacearum* growth and tested purified imizoquins for their effect on bacterial replication. Supporting Figure S11 shows that imizoquin treatment decreases *R. solanacearum* growth modestly at 24 h, with the *rmyA* mutant strain being more sensitive to **1** and **2** than WT (GMI1000). At 48 h, we observed reduced inhibition of *R. solanacearum* growth by imizoquin treatment, except for the case of imizoquin A in the *rmyA* mutant, which could result from variable degradation of the easily oxidizable imizoquins. Nonetheless, these data suggest that imizoquin production favors fungal growth over that of bacteria.

Conclusion

Although genome sequencing reveals that BGCs account for a significant fraction of microbial genomes, biological functions of only a small portion of known BGC-derived metabolites have been explored. Significantly, the mechanisms by which these compounds impact growth and development of other microbes in shared environments is largely uncharted. Here, we characterize alkaloids derived from fungal BGC *imq* and show that they

function as an essential germination stimulatory signal with additional antibacterial properties. Their specific properties suggest that imizoquin and related isoquinolines may provide competitive advantages to *A. flavus* and other closely related fungi in rhizosphere encounters.

The imizoquins appear to promote spore germination through their antioxidant properties. Sensitivity and resistance to ROS was correlated with a loss and overexpression of *imqK* and concomitant loss and increase in imizoquin production. Both exogenous addition of imizoquins and the related isoquinolines TMC-2A/B increased spore germination as did the antioxidant glutathione. ROS-protective properties may be a general feature of quinolones and isoquinolines, as recent preclinical studies with plant isoquinolines such as berberine and neferine have reported on their anti-ROS activities as part of the mechanisms underlying therapeutic efficacy.^{18–20}

Endogenous antioxidants such as the imizoquins may represent developmental cues enabling the fungus to respond to environmental stresses including ROS challenges in a manner ensuring survival. ROSs are known to impact fungal differentiation in several different ways. For example, redox metabolites produced by *Pseudomonas aeruginosa* induce *Aspergillus* sporulation along a diffusion gradient.²¹ ROS synthesis, in association with the RacA GTPase/NADPH oxidase (Nox complex), was found indispensable for phialide elongation and other cellular morphogenesis mechanisms in *A. fumigatus*.²² On the other hand, ROS accumulation can hinder spore germination.^{23–25} In general then, to initiate the process of germination, a set of genes/cellular machinery is required to overcome and adapt to ROS stress. For example, at early stages of germination, conidia express enzymes that are involved in detoxification of ROS,²⁶ and deletion of ROS detoxification genes can result in delayed germination in fungi.²⁷ Furthermore, in *A. nidulans*, deletion of the gene for glutathione reductase (GR), the enzyme required for the production of intracellular reduced glutathione (GSH), resulted in germination defects and an abnormal cell growth pattern that was compensated by adding GSH into the growth medium.²⁸

These examples show that fungi rely on multiple strategies to maintain ROS levels appropriate for spore germination. However, whereas canonical ROS defense response pathways and glutathione generation are conserved in all fungi, ROS protection by secondary metabolites might be species-specific and possibly evolved in response to specific environmental challenges, *e.g.*, competition with bacteria. In addition to our work presented here, other studies have documented inhibition of spore germination by bacterial and plant natural products,^{29,30} including the lipopeptide iturin A.³¹ Thus, it would make sense evolutionarily for fungi to develop spore protective mechanisms and, conversely, for opposing organisms to silence these mechanisms. Considered together, our data suggest that fungal imizoquins represent yet one more ROS protective mechanism that may be particularly responsive to encounters with competing microbes.

METHODS

Fungal and Bacterial Strains and Culture Conditions

All the strains used are listed in Supporting Table S4, and detailed fermentation conditions are in the Methods section of the Supporting Information.

Analytical Methods and Equipment Overview

Detailed information about the instrumentation, software packages, and methods used for NMR spectroscopy, analytical low- and high-resolution HPLC-MS, and semipreparative HPLC for compounds **1–7** is in the Supporting Information.

Gene Cloning, Plasmid Construction, and Genetic Manipulations

Detailed information about the methods used for gene cloning, plasmid construction, and genetic manipulations is in the Supporting Information. The oligonucleotide sequences for PCR primers are given in Supporting Table S5. All strains were verified by PCR and Southern blot analysis (Supporting Figure S12).

Northern Analysis

A total of 50 mL of liquid GMM³² was inoculated with 1.0×10^6 spores (asexual) mL⁻¹ of all appropriate strains in this study and incubated with shaking at 250 rpm at 25 °C. After 48 h, the mycelium was collected, and total RNA was extracted by using Isol-RNA Lysis Reagent according to the manufacturer's instructions (5 Prime).

Germination Assay

Each strain was grown in triplicate in 2 mL of liquid GMM inoculated with 1×10^5 spores mL⁻¹ in a well of a sterile Costar 12-well plate (Corning) and incubated at 30 °C in OKO-Lab microscopic enclosure equipped with a Nikon Eclipse Ti inverted microscope. Germinated spores were observed using a Nikon Plan Fluor 20xPh1 DLL objective and phase-contrast microscope. A spore was considered germinated when the length of the spore protrusion (emerging hyphal tip) exceeded the spore's diameter.^{15,33} Images were captured hourly using the Nikon NIS Elements AR software package (v. 4.13), and the germination rate was determined by counting germlings per hundred spores. The experiment was repeated three times.

Germination Assay with Purified Imizoquins

Compounds **1** and **2** were assessed for their effect on germination of different fungal strains. Each metabolite was dissolved in methanol at a starting concentration of 10 mg mL⁻¹ and stored at -80 °C. Sterile Costar 96 well plates containing 100 μ L of GMM supplemented with various concentrations of purified **1** or **2** (0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM and 1.2 mM) were inoculated at 1×10^5 spores/well. Spores treated with equivalent volumes of the carrier (methanol) served as negative controls for each experiment. The germination rate (%) was measured as above with each treatment assayed in triplicate.

Reactive Oxygen Species (ROS) Stress Assays

Mycelial ROS Assay—To determine the relative tolerance of WT and mutant *imqK* strains to oxidative stress, a range of concentrations of three oxidants was used following the methods of Bok *et al.*³⁴ Briefly, 5 μL of freshly grown (7 days old) 1×10^5 spores mL^{-1} of each strain were inoculated onto solid GMM, which had been supplemented with H_2O_2 (5 μM , 10 μM , or 15 μM), tertiary butyl hydroperoxide (tBOOH; 0.6 mM, 1.2 mM, or 1.8 mM), or menadione (20 μM , 30 μM , or 40 μM). GMM without any stressor served as a control. The plates were incubated at 29 °C, and colony diameters were measured after 3 and 6 days of incubation. All treatments were assayed in triplicate.

Spore ROS Assay—To compare spore viability of *imqK* and OE::*imqK* mutants with WT strain under H_2O_2 (Sigma-Aldrich) stress, the procedure of Qin *et al.* was followed with slight modifications.³⁵ Spore suspensions obtained after 7 days of growth were used to make dilutions in 0.01% Tween 80 in water. These dilutions (1×10^6 spores mL^{-1}) were exposed to different concentrations of H_2O_2 (0, 10, 15, or 30 mM) for 30–90 min. The 30 mM concentration of H_2O_2 gave 50% spore survival after 60 min of incubation and was used in subsequent assays. After incubation for 60 min at 29 °C, spores were washed twice by centrifugation (1500g for 2 min) and resuspension in 1 mL of distilled H_2O . The washed spores were diluted, and 100 μL of 1×10^3 spores mL^{-1} were spread on GMM plates. Plates were incubated at 29 °C and observed for the number of colonies formed after 24 and 48 h. Three replicates were performed for all conditions.

Germination ROS Assay—To estimate the effect of H_2O_2 on germination of mutant and WT strains, 100 μL of GMM, containing freshly harvested spores (1×10^5 spores mL^{-1}), was treated with different concentrations of H_2O_2 (10^{-12} to 10^{-2} M) in triplicate in sterile Costar 96 well plate at 30 °C. GMM without H_2O_2 was used as a control. The germination rate (%) was measured for the 9 h time point.

For one concentration of H_2O_2 (0.7 mM), three different concentrations of imizoquin A (0.1 mM, 0.2 mM, 0.4 mM) were added to the treatment to assess germination. Treatment with H_2O_2 only was the positive control, and treatment with either **1** or H_2O_2 was used as the negative control. Each treatment was performed in triplicate.

Antioxidant Assay

ROS quenching potential of the imizoquins was analyzed using the OxiSelect Total Antioxidant Capacity Assay Kit (Cell Biolabs) following the manufacturer's instructions. Briefly, 20 μL of diluted uric acid standards (0–1 mM) and imizoquins (0–1 mM) were combined with 180 μL of 1 $\times$ reaction buffer/well in a Costar 96 well plate. After mixing thoroughly, an initial absorbance was obtained at 490 nm using a BioTek EPOCH 2 microplate reader with Gen5 acquisition software. Then, 50 μL of the 1 $\times$ copper ion reagent was added into each well, and the plate was incubated for 5 min on an orbital shaker. To terminate the reaction, 50 μL of 1 $\times$ stop solution was added, and the final absorbance was measured again at 490 nm.

Germination Assay with Antioxidant

Conidia suspensions of *imqK* mutants and WT strain (1×10^5 spores mL⁻¹ of water containing 0.01% Tween 80) were treated with three different concentrations (0.1 mM, 0.2 mM, or 0.4 mM) of glutathione (GSH) in GMM and incubated at 30 °C. The number of germinated spores out of 100 spores was counted hourly 5–10 h postinoculation (hpi). Three independent replicates were analyzed for each treatment.

Aspergillus/Ralstonia Interactions

Metabolite extraction of *R. solanacearum* strains (GMI1000 and *rmyA*) was carried out following the protocol of Spraker *et al.*⁶ Briefly, overnight liquid cultures of both GMI1000 and *rmyA* strains were inoculated into 500 mL of CPG containing 10 g of Amberlite XAD-16 resin (Sigma-Aldrich) for 3 days at 30 °C and 200 rpm. The resin was then vacuum filtered and washed twice with 500 mL of double-distilled H₂O to remove cellular debris and excess salts. The adsorbed metabolites were then eluted with 1:1 methanol/dichloromethane, and the organic fraction was dried using vacuum centrifugation (Buchi R-210 Rotary Evaporator, Vacuum Controller V-500). Subsequently, these crude extracts were reconstituted in 100% methanol.

The germination assay was used to test the impact of the crude extracts of GMI1000 and *rmyA* on the growth of WT and mutant *imqK* strains. Each test strain, inoculated in 100 μ L of GMM, was treated with 10 μ L of the crude methanolic extract in well of a sterile Costar 96 well plate maintained at 30 °C. Tests were performed in triplicate, and 10 μ L of methanol served as the control treatment. Due to the solvent effect, germination rate was measured hourly at 11–16 h of incubation.

Growth Inhibition of *R. solanacearum* in the Presence of Imizoquins

Overnight liquid cultures of *R. solanacearum* strains (GMI1000 and *rmyA*) were pelleted by centrifugation at 11,000 rpm, washed twice in equal volumes of sterilized, double-distilled water, quantified, and adjusted to an OD₆₀₀ of 0.1. Imizoquin A (**1**) and imizoquin B (**2**) were added to CPG medium (0.4 mM final concentration of each metabolite) in wells of a sterile Costar 96 well plate. Then, 5 μ L of bacterial suspension was added in each well to make a final volume of 100 μ L/well. An equal volume of methanol in CPG was used as a control. OD₆₀₀ and OD₄₅₀ were measured after 0, 12, 24, and 48 h of incubation at 30 °C. Measured values from time 0 were subtracted from subsequent time point values to normalize for absorbance by imizoquins.

Statistical Analyses

Statistical differences of data were analyzed using the GraphPad Prism 7 software package (GraphPad Software Inc.). Students t-tests were used for pair-wise comparisons, and one-way ANOVA were used for multiple comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparative transcriptomic analysis and *imq* cluster annotation. (a) Differential gene expression (schematic, for data see Supporting Table S1) in *A. flavus* treated with WT (GMI1000) or *rmyA* *R. solanacearum* extracts. (b) *imq* gene cluster and putative assignments: Hyp, hypothetical protein; NRPS, nonribosomal peptide synthetase (A = adenylation, T = thiolation, C = condensation, TE = thioesterase domain); MO, phenol 2-monooxygenase; Trs, transporter; GOX, gibberellin oxidase; N-MT, N-methyltransferase; O-MT, O-methyltransferase; FAD, FAD-binding domain protein; TF, transcription factor. **A. flavus* produces chlamydospores—the oval structures in the diagram—in response to ralstonin containing extracts from WT *R. solanacearum*.

Figure 2.

Imq cluster expression and metabolite identification and characterization. (a) Northern blot analysis of *imq* cluster and flanking genes in *OE::imqK* and WT *A. flavus* strains. (b) LC-MS extracted ion chromatograms for **1** and **2** in extracts of *OE::imqK* and WT. (c) Structures of imizoquins A–D, **1–4**, and previously characterized TMC-2A/B (**5, 6**). Key COSY correlations (blue double-headed arrows) and key HMBC correlations from each proton to the indicated carbon (red single-headed arrows) are shown for **1** and **2**.

Figure 3.

Proposal for imizoquin biogenesis. ImqB (NRPS, green), ImqF (N-methyltransferase, pink), ImqC (phenol 2-monooxygenase, purple), ImqG (O-methyltransferase, yellow), ImqH (FAD-dependent oxidase, blue), ImqE (gibberellin oxidase, turquoise), A (adenylation domain), T (thiolation domain), C (condensation domain), TE (thioesterase domain), and [Ox.] (oxidation).

Figure 4.

Imizoquins' promotion of germination and provision of resistance to ROS. The germination rate of *imqK* strains was tested between 4 and 9 h postinoculation (hpi) in glucose minimal media. Appearance of a germ tube indicated the onset of germination. (a) The *imqK* mutant shows a significantly lower rate of germination, whereas the OE::*imqK* mutant shows a significantly higher rate of germination compared to the WT strain. (b) Purified imizoquins enhance germination rate in WT. Increasing the imizoquin concentration significantly increases germination rates as compared to control. (c and d) Imizoquin A and B restore germination in the *imqK* mutant in a dose-dependent manner. (e) Growth of *imqK* mutants and WT in GMM media with added H₂O₂, *t*-butyl hydroperoxide (tBOOH), and menadione. Following incubation at 30 °C for 3 days, colony diameters were measured. (f) Survival of conidia of *imqK*, OE::*imqK*, and WT was tested by incubating spores of each strain with 30 mM of H₂O₂. Colonies were counted after incubation at 30 °C for 24 h. Values represent the average of three trials with error bars indicating standard error. The Student's *t* test was used to assess statistical significance; **p* value < 0.05; ***p* value < 0.01; ****p* value < 0.001.

Figure 5.

Antioxidant properties of the imizoquins. (a) The *imqK* and (b) WT strains were treated with different concentrations of **1** in the presence of 0.7 mM H_2O_2 , and their germination rate was measured over time. Imizoquin A (**1**) counteracted the germination inhibitory activity of H_2O_2 . Statistical significance is indicated as compared to positive control. (c, d) Glutathione (GSH) was examined for its effect on germination in the *imqK* mutant and WT. All tested concentrations of GSH significantly promoted germination rate in the *imqK* mutant and WT. Values represent means of three replicates $\pm$ standard errors. Asterisks indicate statistical significance according to one-way ANOVA followed by Tukey's posthoc test; **p* value < 0.05, ***p* value < 0.01, ****p* value < 0.001.

Figure 6.

R. solanacearum extracts (GMI1000 and *rmyA*) affecting *A. flavus* germination. Conidia suspensions (1×10^5 spores mL^{-1}) of different *A. flavus* strains were treated with extracts of WT *R. solanacearum* (GMI1000) and the *rmyA* mutant. Germination rates were assessed 11–16 h postinoculation. (a) In WT *A. flavus*, extracts from the *rmyA* mutant significantly enhanced germination rates at all time points, whereas GMI1000 extracts decreased germination rates at earlier and increased rates at later time points. (b) OE::*imqK* of germination rates are similar to WT strain. (c) For the *imqK* mutant, *rmyA* extracts promoted germination at later time points, whereas GMI1000 extracts consistently delayed germination. Values represent the average of three trials $\pm$ standard error. Asterisks indicate the statistical significance (relative to the control) according to the Student's *t* test; **p* value < 0.05; ***p* value < 0.01; ****p* value < 0.001.