



GH25 lysozyme mediates tripartite interkingdom interactions and microbial competition on the plant leaf surface

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Microbial communities inhabiting plants have emerged as crucial factors in regulating plant health and defense against disease-causing pathogens. The yeast *Moesziomyces bulbatus* ex. *Albugo* (MbA) on *Arabidopsis* produces a glycoside hydrolase 25 (GH25) protein that regulates the leaf microbiome by antagonizing the pathogenic oomycete *Albugo laibachii*. Applying MbA or GH25 rescued *Arabidopsis thaliana* shoot fresh weight under *A. laibachii* infection, highlighting their crop protection potential. Interaction assays revealed no antagonistic activity of GH25 against other plant pathogenic oomycetes or fungi besides *A. laibachii*. We identified a community of bacteria closely associated with *A. laibachii*. Three of these bacteria are inhibited by GH25 and one of them, *Curtobacterium* sp. could override the inhibition of *A. laibachii* by MbA. This points to a tripartite antagonism where *Curtobacterium* and *A. laibachii* protect each other from MbA. Moreover, *Curtobacterium* selectively inhibits other *A. laibachii*-associated bacteria not targeted by MbA but that themselves suppress *A. laibachii*. This study uncovers an interkingdom network where GH25 lysozyme shapes microbial interactions between yeast, oomycete, and associated bacteria.

microbial competition | *Albugo laibachii* | GH25 lysozyme | phyllosphere | moesziomyces

Pathogenic microorganisms such as bacteria, fungi, oomycetes, and viruses cause various plant diseases. In particular, oomycete and fungal pathogens infect several agronomically important crops; thereby, posing a threat to global food security. For example, the oomycete *Phytophthora infestans* caused the Irish famine (1845) through potato late blight disease, while other species of *Phytophthora*, namely *Phytophthora ramorum*, *Phytophthora capsici*, and *Phytophthora sojae* are known to infect oak trees, solanaceous crops, and soybean, respectively (1). *Botrytis cinerea*, a fungal pathogen with a broad host range, can cause severe economic losses with the disease control being heavily dependent on fungicide application (2, 3). In addition, climate change is predicted to exacerbate plant disease outbreaks through the emergence and evolution of pathogenic strains, potentially affecting both natural biodiversity and agricultural systems (4).

Microbial antagonism or biological control of pathogens by the action of beneficial microorganisms has emerged as an effective and sustainable approach toward plant protection. For example, biological control of *Phytophthora* has been reported by inhibitory activities of potato associated bacteria (5), or by volatiles secreted from bacteria (6) and fungi (7). Therefore, a thorough knowledge on the mechanism of microbial antagonism is necessary to attain successful crop protection. While several modes of action for microbial antagonists have been described, such as triggering of plant immune response, hyperparasitism on pathogens, or secretion of secondary metabolites (8), competition by associated microbiota may be a crucial criterion for effective pathogen control and restoration of plant health (9). Among such interactions, *Albugo laibachii*, the causal agent of white rust, has emerged as a microbial hub which promotes the dysbiosis of *Arabidopsis* phyllosphere (10).

Microbiota inhabiting plants secrete a range of glycoside hydrolases (GH) which aid nutrient acquisition and competition with other microbes, or can be virulence factors to colonize a host (11). Antagonistic fungi such as *Trichoderma* were reported to produce a variety of hydrolytic enzymes to antagonize fungal pathogens in crop plants (12). For example, chitinase from *Trichoderma asperellum* PQ34 inhibited growth of fungal pathogens *Sclerotium rolfsii* and *Colletotrichum* (11, 13). Additionally, cellulose encoding genes from *Trichoderma harzianum* were found to elicit plant immunity against pathogen *Fusarium graminearum* by upregulating the production of DIMBOA and other defense related genes in maize roots (14).

GH are one of the largest and most diverse groups among hydrolytic enzymes with 172 families and 18 different GH clans (15). In phytopathogenic fungi, GH families have

Significance

Plant-associated microbial communities play a key role in plant health and disease resistance. This study uncovers a tripartite interkingdom interaction between a basidiomycete yeast, an oomycete pathogen, and a commensal bacterium. The observed antagonism is mediated by a glycoside hydrolase 25 (GH25) lysozyme secreted by yeast, which selectively inhibits a bacterial key microbial associate of the pathogen. Our findings provide intricate mechanistic insight into the dynamics of the microbiota thriving on leaf surfaces and suggest strategies for disease control through microbiome modulation.

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been characterized mostly with respect to triggering of plant immunity and pathogenic virulence. For example, secreted GHs from oomycete *P. sojae* and fungi *Verticillium dahliae*, *Magnaporthe oryzae*, and *Fusarium oxysporum* can act as microbe-associated molecular patterns (MAMPs) (16–19) or release damage associated molecular patterns (DAMPs) leading to the activation of pattern triggered immunity, the first layer of plant defense responses (11). In contrast, deletion of cell wall hydrolyzing enzymes encoded by GH families in *B. cinerea*, *M. oryzae*, and *Alternaria alternata* impairs fungal virulence (16–18).

GH can also be involved in microbial antagonism of pathogens. For example, deletion of the GH18 chitinase gene in the fungus *Clonostachys rosea* reduced inhibitory activity against *B. cinerea* and *Rhizoctonia solani*, although biocontrol of *B. cinerea* was not compromised (11, 20). More recently, Glycoside Hydrolase 30 produced by the bacterium *Bacillus paralicheniformis* triggered plant defense responses via programmed cell death and restricted phytopathogens such as *Sclerotinia sclerotiorum*, *Phytophthora capsici*, and tobacco mosaic virus (21). In addition, GH19 chitinase from *Chitinilyticum aquatile* CSC-1 was recombinantly expressed in *Escherichia coli* and displayed inhibition of fungal growth in several *Fusarium* species, suggesting its role in biological control of phytopathogenic fungi (22).

In previous work (23) we described how the basidiomycete yeast, *Moesziomyces bullatus* ex. *Albugo* on *Arabidopsis* (*MbA*) regulates the *Arabidopsis thaliana* phyllosphere microbiota by inhibiting the white rust pathogen *A. laibachii* through a secreted Glycoside Hydrolase 25 (GH25) protein. The GH25 family of hydrolases, characterized by a DXE motif active site, cleaves the β -1,4-glycosidic linkage between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in the bacterial peptidoglycan (24). However, the biological function of GH25 in fungi remains poorly understood.

In this study, we functionally investigated how GH25 antagonizes *A. laibachii*. We examined the effect of *MbA* and *MbA*_GH25 against other plant pathogens and found that GH25 acts specifically against *A. laibachii* through an indirect mechanism involving an associated bacterium of the genus *Curtobacterium*. We extended our analysis by functionally characterizing GH25 orthologs from the plant pathogenic fungi *Ustilago maydis* and *R. solani* in the antagonism toward *A. laibachii* and suggest a possible mechanism of evolutionary adaptation of GH25 specificity.

Results

***MbA* Limits Growth of *A. laibachii* through GH25 Activity.** After we had explored how a basidiomycete yeast, *MbA* antagonizes the oomycete *A. laibachii* in the *A. thaliana* phyllosphere by secreting GH25 (23), we aimed to understand how GH25 mediates the previously observed antagonism. We first quantified *A. laibachii* infection symptoms in 3-wk-old *A. thaliana* seedlings grown in a gnotobiotic plate system with and without application of *MbA* (Fig. 1A). Additionally, we quantified the relative fungal biomass of *A. laibachii* at 10 dpi by normalizing the oomycete internal transcribed spacer (ITS) 5.8s sequence to the *A. thaliana* housekeeping gene *Efl- α* . We observed that *A. laibachii* biomass was also significantly reduced in the presence of *MbA* (Fig. 1B). Moreover, a protective effect of *MbA* on the plant was evaluated by measuring the fresh shoot weight of *A. thaliana* seedlings following different treatments (Fig. 1C). We observed that the reduced fresh shoot weight during oomycete infection could be reversed by pretreating the plants with *MbA* or by adding recombinantly produced *MbA*_GH25 to the yeast deletion strain *MbA* Δ gh25 (Fig. 1C).

Since *MbA*_GH25 plays a key role in oomycete inhibition, we analyzed the evolutionary conservation of GH25 in the fungal kingdom. Previously, we showed that the DXE active site motif is highly conserved in the GH25 amino acid sequence of different fungal groups such as Basidiomycetes, Ascomycetes, and Chytrids (23). A constructed phylogenetic tree including various fungal species showed that the GH25 of Ustilaginales, including *MbA* and *U. maydis*, clustered together with a high degree of sequence similarity. However, evolutionarily more diverse ascomycete fungi, including several plant pathogens, also have significantly conserved GH25 orthologs (Fig. 2A). The orthologs showed a high sequence similarity and structural conservation (*MbA* vs. *U. maydis*: perc. identity: 77.87%, rmsd 0.243; *MbA* vs. *R. solani*: perc. identity: 51.25%, rmsd: 0.481) (Fig. 2C and *SI Appendix*, Fig. S1). We recombinantly expressed the three orthologs from *MbA*, *U. maydis*, and *R. solani* in *U. maydis* wild type strain FB1. Similar expression levels in the recombinant *U. maydis* strains were validated via qPCR (*SI Appendix*, Fig. S1). The recombinant strains were then inoculated onto *A. thaliana* seedlings prior to *A. laibachii* inoculation and infection symptoms on leaves were scored at 11 dpi. FB1 strains overexpressing active GH25s from *MbA* or *U. maydis* significantly inhibited *A. laibachii* infection *in-planta*, as opposed to GH25 from *R. solani* (Fig. 2B). Therefore, inhibitory activities of GH25 from *MbA* and *U. maydis* are functionally conserved compared to GH25 from the more distantly related fungal species *R. solani*.

No Evidence of Plant Immunity Activation or Direct Inhibition of Oomycete and Fungal Plant Pathogens by *MbA* GH25. To elucidate the mechanism of the observed *MbA* antagonism toward *A. laibachii*, we explored whether *MbA*_GH25 impacts plant immunity. To this end, *A. thaliana* seedlings were treated with purified recombinant *MbA*_GH25, *MbA*_GH25 (D124A), or heat-inactivated (Hi) *MbA*_GH25 proteins as a negative control. The broadly recognized bacterial MAMP flagellin 22 (flg22), was used as a defense-inducing positive control in the elicitor assay. Thirty minutes and six hours after treatments, seedlings were harvested to check for activation of several defense marker genes. Flg22 treatment induced the expression of plant defense genes *WRKY53*, *WRKY33*, *WRKY30*, and *FRK1* compared to other treatments (*SI Appendix*, Fig. S2). For both time-points, only slight PR-gene upregulation by GH25 treatment could be observed with no difference in defense gene expression levels detected between active and inactive (mutated and heat-inactivated) versions of the *MbA*_GH25 protein (*SI Appendix*, Fig. S2). Therefore, we concluded that the enzymatic activity of *MbA*_GH25 likely does not induce plant defense in *A. thaliana*.

Simultaneously, we explored whether *MbA*_GH25 directly targets the oomycete. The cell walls of oomycetes are mainly composed of β -1,3, and β -1,6 glucans (25), with varying levels of NAG-the building blocks of bacterial peptidoglycan (26). Therefore, to determine whether *MbA*_GH25 directly targets *A. laibachii* cell wall we used commercially available Laminarin (seaweed polysaccharide with β -1,3 linked glucan bonds and β -1,6 linked side chains) as a substrate to monitor for release of oligosaccharides upon addition of purified *MbA*_GH25 protein via Thin Layer Chromatography. This approach showed no activity of GH25 on Laminarin (*SI Appendix*, Fig. S3A). Additionally, we isolated *A. laibachii* cell walls from harvested zoospores (26) and looked for activation of *MbA* *gh25* gene expression. However, no significant induction of the *gh25* expression was detected in growing *MbA* cultures (*SI Appendix*, Fig. S3B). Thus, we could not gain experimental evidence for a direct inhibition of *A. laibachii* by *MbA*/GH25.

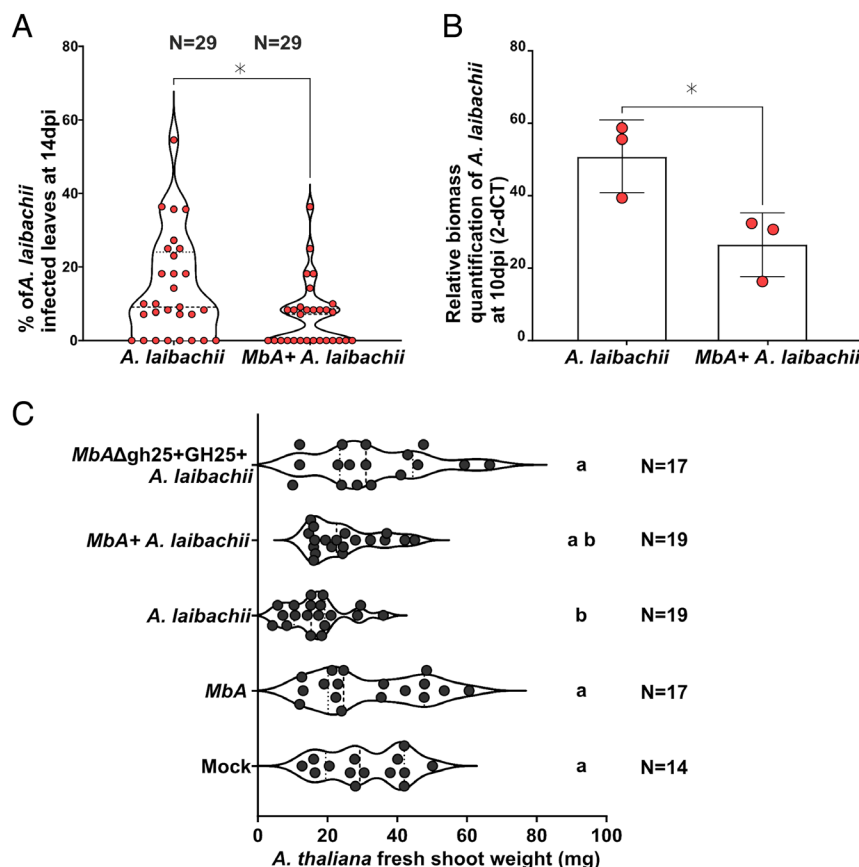


Fig. 1. *MbA* restricts *A. laibachii* growth and recovers *A. thaliana* fresh shoot weight during infection. (A) Addition of *MbA* reduces *A. laibachii* induced leaf disease symptoms of *A. thaliana* at 14 dpi. The number of infected plants (N) were scored across three biological replicates (Mann-Whitney test, P value = 0.04). (B) Relative quantification of *A. laibachii* biomass in response to *MbA* treatment by qPCR. The oomycete ITS 5.8 s was normalized to *A. thaliana* EF1- α gene to quantify the amount of *A. laibachii* DNA in the samples at 10 dpi (unpaired t test, P value = 0.03). Error bars indicate SD. (C) Fresh shoot weight measured at 11dpi showed *A. thaliana* seedlings to be reduced in growth upon infection with *A. laibachii* compared to mock treated seedlings. Addition of purified *MbA*_GH25 protein to the *gh25* deletion mutant of *MbA* (*MbAΔgh25*) led to a higher recovery of fresh shoot weight in *A. laibachii* infected seedlings. The number of seedlings (N) were measured across three biological replicates. One-way ANOVA and Tukey's HSD (multiple comparisons of means; 95% family-wise confidence level) was performed to find significant difference between treatments.

To determine whether the antagonistic activity of *MbA*/GH25 is specific to *A. laibachii*, we tested for possible interactions with the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*; biotrophic pathogen of solanaceous plants, not adapted to *A. thaliana*) as well as the fungal pathogen *B. cinerea* (necrotrophic pathogen, broad host range). To test for *MbA*/GH25 effects on *Hpa* infection, *A. thaliana* seedlings (Col-0 and Col-0 *eds1-12*) (27) were pretreated with *MbA* and *MbAΔgh25*, followed by inoculation of *Hpa* 2 d later. No significant differences in *Hpa* sporulation (release of *Hpa* spores per gram of *A. thaliana* at 5 dpi) were observed among the different treatments in both *A. thaliana* wild-type Col-0 and the *Hpa*-hypersusceptible mutant *eds1-12* (Fig. 3A). *Hpa* sporulation was significantly higher in the *eds1-12* mutant line compared to Col-0, indicating that *Hpa* infection conditions on *A. thaliana* were suitable. (Fig. 3A). Furthermore, relative *gh25* expression levels were quantified in *MbA* upon interaction with *Hpa* on *A. thaliana* and no significant change in *gh25* expression was observed (SI Appendix, Fig. S4A). Thus, the presence of the basidiomycete yeast or its active and inactive hydrolase did not impact leaf infection *Hpa*.

In-plant interaction assays of *P. infestans* with *MbA* were performed using detached leaves of 5-wk-old *Nicotiana benthamiana*. We observed that necrotic lesions caused by *P. infestans* were not reduced upon its coinoculation in the presence of *MbA* and *MbAΔgh25* (Fig. 3B). In addition, the *MbA gh25* gene was

recombinantly expressed in the smut pathogen *U. maydis* [strain SG200 (28)] and tested for interaction with *P. infestans* in the detached leaf assay. However, coinoculation of the recombinant strain SG200_pOtef:*MbAGH25* that overexpressed *MbA* GH25 did not limit the development of *P. infestans* induced necrotic lesions (Fig. 3B).

Confrontation assays in axenic culture between *MbA* strains and *P. infestans* were carried out on Rye-Sucrose Agar plates. However, although no zone of inhibition was observed between the two interacting microorganisms, *P. infestans* was restricted from growing over the area already colonized by yeast strains (SI Appendix, Fig. S4B). To investigate the inhibitory effect of *MbA* on a necrotrophic fungus, *B. cinerea* was tested by using both in-vitro and in-planta assays. During the axenic confrontation assay, no inhibition of *B. cinerea* could be observed upon confrontation with *MbA*, *MbAΔgh25*, and purified *MbA*_GH25 protein (SI Appendix, Fig. S4C). However, the lesion size of *B. cinerea* was significantly reduced after pretreatment of *A. thaliana* with both *MbA* and *MbAΔgh25*, indicating an inhibition of *B. cinerea* by *MbA* that is independent of GH25 activity (Fig. 3C). Thus, the observed antagonistic activity of *MbA* via GH25 appears to specifically act against *A. laibachii*.

In summary, we found no evidence that GH25 stimulates plant immunity or exhibits direct antimicrobial activity against any of the tested eukaryotic plant pathogens.

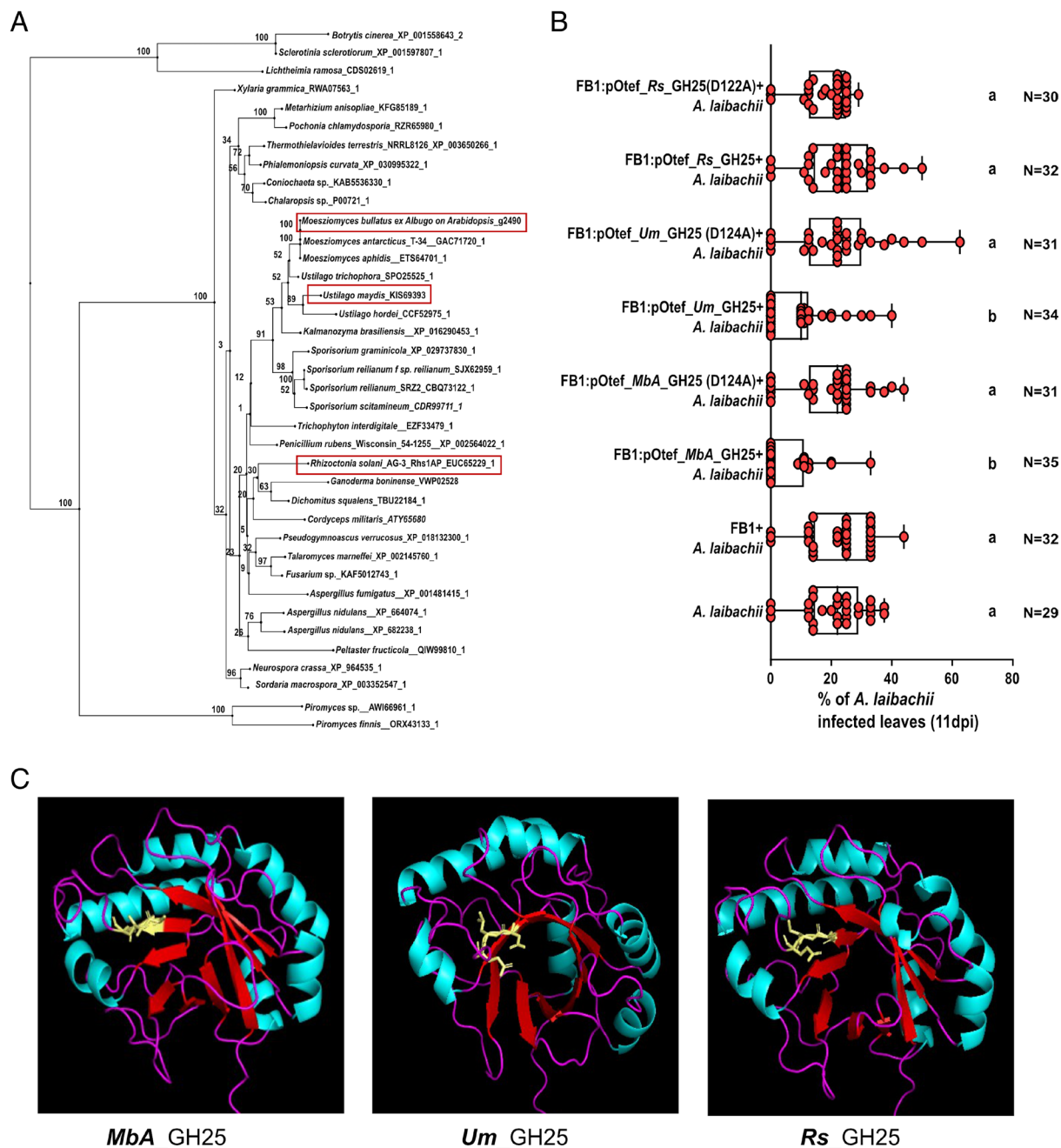


Fig. 2. Overexpression of GH25 orthologs from MbA and *U. maydis* restricts *A. laibachii* growth compared to GH25 from distantly related *R. solani*: (A) Phylogenetic tree of GH25 orthologs. Protein sequences were aligned using MAFFT version 7 multiple alignment program for amino acid of nucleotide sequences with default parameters. Phylogenetic tree construction was based on the alignment using Phylo.io. MbA, *U. maydis*, and *R. solani* GH25 are marked by red boxes. (B) Infection assay of *A. laibachii* on *A. thaliana* with pretreatment of *U. maydis* wild type strain FB1 and recombinant FB1 strains overexpressing GH25 orthologs from MbA, *U. maydis* (Um), and *R. solani* (Rs) (FB1_pOtef_*MbA/Um/Rs*_GH25) in active or mutant version (D124A). % of *A. laibachii* infected leaves were scored at 11 dpi across three biological replicates in nonsterile condition. Kruskal–Wallis or Dunn’s multiple comparisons test (alpha 0.05) was performed to find significant difference between treatments. N = number of seedlings analyzed. (C) 3-D structures of GH25 orthologues obtained by AlphaFold 3 and visualized by pyMOL, showing active site motif (yellow) in the center.

Bacteria Associated with *A. laibachii* Are Targeted by GH25. Recently, Goossens et al. (2023) investigated the presence of several bacterial isolates closely associated with *H. arabidopsidis* on *A. thaliana*. The bacterial population thrived during *Hpa* infection and was actively recruited by the plant to limit disease progression (29). Therefore, we hypothesized that GH25-mediated inhibition of *A. laibachii* might be mediated. We tested whether *A. laibachii* could be associated with a bacterial community. In gnotobiotic *A. laibachii* infection assays, oomycete zoospores are treated with an antibiotic cocktail (streptomycin, kanamycin, rifampicin, and geneticin)

prior to inoculation of *A. thaliana* seedlings. To determine whether any bacterial isolates remained after the antibiotic treatment, we harvested zoospores of *A. laibachii* NC14 strain and plated them on King’s B medium after treatment with the antibiotic cocktail. This treatment resulted in the isolation of a set of *A. laibachii*-associated bacteria, comprising mainly *Pseudomonas* sp., *Microbacterium* sp., and *Curtobacterium* sp. (SI Appendix, Fig. S5A and Dataset S2). The isolates were tested for antibiotic resistance against all previously treated antibiotics as well as widely used carbenicillin as a control antibiotic (SI Appendix, Fig. S5B). No isolate was resistant to all

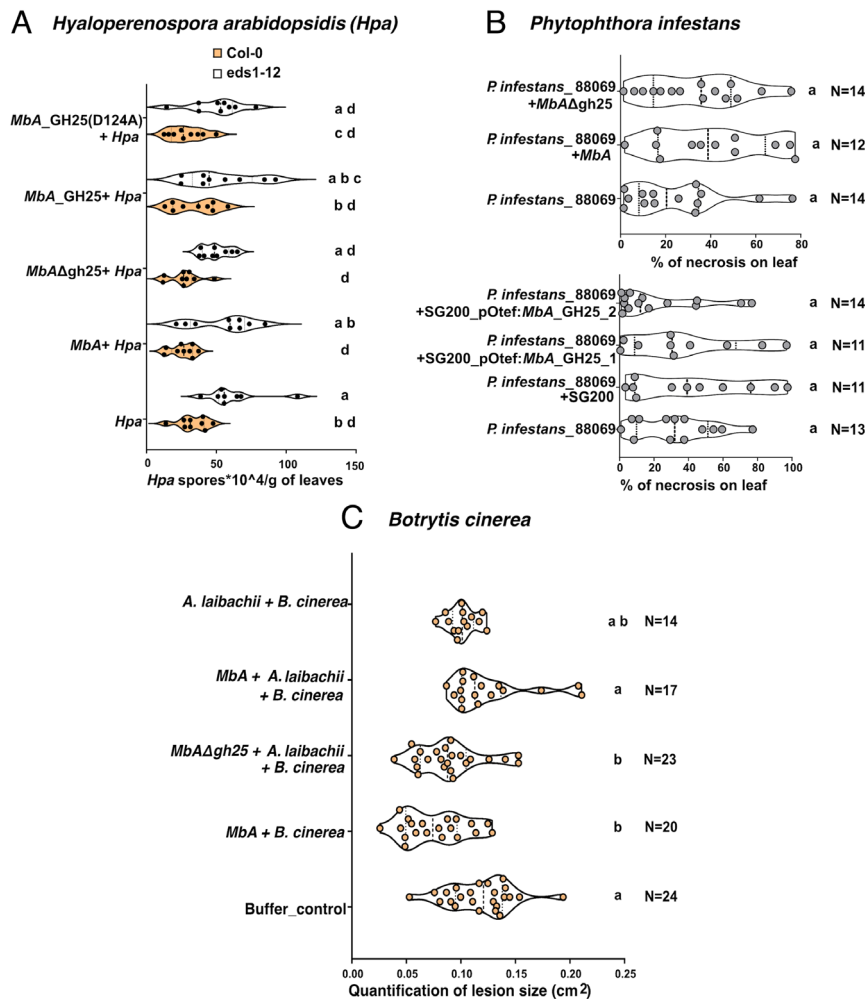


Fig. 3. GH25-mediated inhibition of *A. laibachii* is not conserved against other pathogenic oomycetes and fungi. (A) *MbA* and purified *MbA_GH25* do not affect *Hpa* infection in *A. thaliana* Col-0 and *eds1-12* mutant (Col-0 background). Experiments were conducted in three biological replicates (consisting of three technical replicates). Quantification of *Hpa* spores * 10⁴/g of leaves was performed at 5 dpi. Two-way ANOVA and Tukey's multiple comparisons test was performed to find significant differences. (B) Interaction of *P. infestans* with *MbA* strains and *U. maydis* strains: Quantification of necrotic area on leaf surface was performed by ImageJ and percentage of necrosis calculated in three biological replicates (N = number of leaves analyzed). One-way ANOVA and Tukey's multiple comparisons test (alpha 0.05) was performed to find significant difference between treatments. (C) In planta droplet infection assay was performed by dropping 5 μ L of spore suspension in detached *A. thaliana* leaves, which were pretreated with mock (water), *MbA*, *MbAΔgh25*, *MbA* + *A. laibachii* or *A. laibachii*. Lesion size quantification was performed using ImageJ across three biological replicates (N = number of lesions analyzed). One-way ANOVA and Tukey's multiple comparisons test (alpha 0.05) was performed to find significant difference between treatments.

antibiotics, while some strains like *Curtobacterium* sp. were resistant to none of the treated antibiotics. These results indicate a close association of isolated strains and zoospores.

We then tested the individual *A. laibachii* associated bacterial strains in one-to-one interactions with *MbA*, purified *MbA_GH25* protein, or a recombinant *U. maydis* strain constitutively expressing *MbA_GH25* (Fig. 4 and SI Appendix, Fig. S5 C and D). *MbA* inhibited two of the *Pseudomonas* strains, while the recombinant *MbA_GH25* strain inhibited two of the *Curtobacterium* strains (SI Appendix, Fig. S5). Since *MbA* does not express GH25 on plate, we further used purified *MbA_GH25*, which inhibited both *Staphylococcus* sp. and *Curtobacterium* isolates on-plate. In contrast, the catalytically inactive *MbA_GH25* (D124A) protein did not cause any inhibition, indicating that the antibacterial activity is dependent on GH25 enzymatic activity (Fig. 4 A–C). To assess whether GH25 acts bactericidal (agent that kills bacteria) or bacteriostatic (agent that inhibits growth and reproduction of bacteria) on the *A. laibachii* associated bacteria, we performed bacterial cell viability assays. Active GH25 could significantly reduce *Curtobacterium* sp.#2 and *Curtobacterium* sp. #1 viable cell numbers, whereby *Staphylococcus*

sp. viability was unaffected (Fig. 4D). Hence, GH25 acts bactericidal on *Curtobacterium* sp., making *Curtobacterium* sp. the potential main GH25 target. *Curtobacterium* sp. #2 and *Curtobacterium* sp. #1 clustered within the same group when all strains were analyzed together by two-way ANOVA, reflecting their similarly strong inhibition. However, a direct pairwise comparison suggested a stronger survivability reduction of *Curtobacterium* sp. #2 compared to *Curtobacterium* sp. #1 (SI Appendix, Fig. S6). This trend was supported by a complementary CFU assay, in which *Curtobacterium* sp. #2 survivability reduction was significantly stronger compared to *Curtobacterium* sp. #1 (SI Appendix, Fig. S6). In addition, we performed a bacterial lysis assay on both *Curtobacterium* sp. strains and the commercial *Micrococcus lysodeikticus* which showed cell lysis reflected by reduced optical density for both GH25 and a commercial lysozyme (SI Appendix, Fig. S7), further demonstrating the lysozyme activity of GH25 on *Curtobacterium* sp. Thus, *A. laibachii* is associated with a community of bacteria of which *Curtobacterium* sp. is inhibited by *MbA*/GH25 activity. Next, we tested whether these microbial interactions are linked with the antagonistic interplay of *MbA* and *A. laibachii* on the leaf surface.

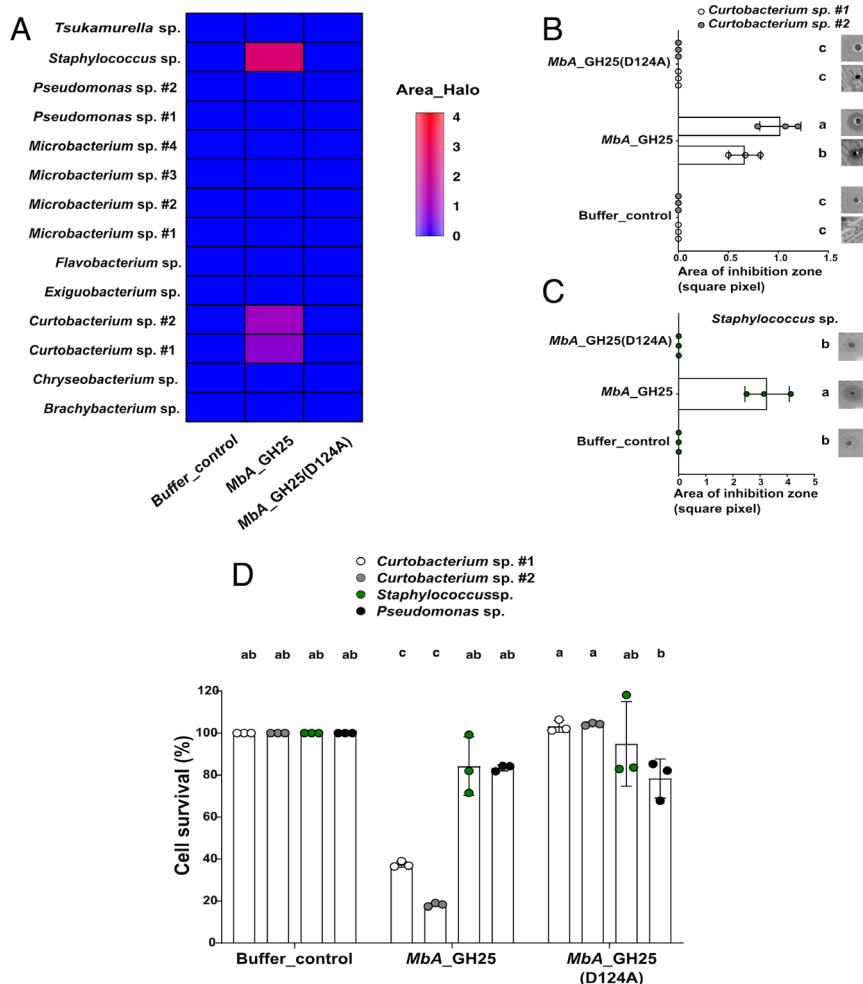


Fig. 4. *A. laibachii* associated bacteria are targeted by MbA_GH25. (A) Heatmap showing overview of the confrontation assay between *A. laibachii* associated bacteria and purified recombinant MbA_GH25/MbA_GH25(D124A) protein across three independent replicates. *Curtobacterium* sp. (#1 and #2) and *Staphylococcus* sp. were inhibited by MbA_GH25 active protein. (B and C) Zone of inhibition of both isolates of (B)-*Curtobacterium* sp. and (C)-*Staphylococcus* sp. by purified MbA_GH25 was analyzed compared to buffer control and mutated MbA_GH25 (D124A) across three independent replicates. Representative image of bacterial inhibition added next to respective treatments as indicated by the presence or absence of halo formation surrounding the agar well, 3 d after application of recombinant protein (0.8 $\mu\text{g}/\mu\text{L}$). Error bars show SD. Two-way ANOVA and Tukey's multiple comparisons test (alpha 0.05) was performed to find significant difference between treatments. (D) Cell viability luciferase assay of *Curtobacterium* sp., *Staphylococcus* sp., and *Pseudomonas* sp. to analyze % of bacterial cell survival upon treatment with MbA_GH25, MbA_GH25 (D124A), and buffer_control across three independent replicates. Two-way ANOVA and Tukey's multiple comparisons test (alpha 0.05) was performed to find significant difference between treatments.

An Antagonistic Interplay between Bacteria, an Oomycete, and a Fungal Yeast. To elucidate an eventual biological role of the observed microbial interplay, the tripartite interactions of MbA, *A. laibachii*, and either *Curtobacterium* sp., *Pseudomonas* sp., or *Staphylococcus* sp. on *Arabidopsis* leaves were investigated. While *Pseudomonas* sp. and *Staphylococcus* sp. had no, or marginal negative impact on *A. laibachii*, oomycete infection was strongly influenced by presence of *Curtobacterium* sp. (Fig. 5 A–C). *Staphylococcus* sp. inhibited *A. laibachii* infection both in the presence and absence of MbA, similar to *Curtobacterium* sp. #1 (Fig. 5C). In contrast, *Pseudomonas* sp. had no inhibitory effect on *A. laibachii* infection and could not significantly suppress the inhibition of *A. laibachii* by MbA (Fig. 5C). Interestingly, the two strains of *Curtobacterium* sp. showed contrasting behavior in the tripartite interaction: *Curtobacterium* sp. #1 slightly reduced *A. laibachii* infection (Fig. 5A). In contrast, *Curtobacterium* sp. #2 did not significantly affect *A. laibachii* infection. Moreover, coinoculation almost completely restored *A. laibachii* infection in the presence of MbA; i.e., *Curtobacterium* sp. #2 protected *A. laibachii* from antagonism by MbA (Fig. 5B). Since *Curtobacterium* sp. #2 was identified as the most impacted strain by GH25 presence, we

mostly focused on the interaction with this strain in further experiments.

Next, we performed a qPCR approach to quantify the growth of the interacting microbes on the plant surface. This revealed that MbA biomass was reduced in the presence of *Curtobacterium* sp. #2 (Fig. 5E). Also, growth of *Curtobacterium* sp. #2 was significantly reduced in the presence of MbA (Fig. 5D), reflecting a bidirectional antagonism between *Curtobacterium* sp. #2 and MbA. Notably, the MbA-mediated inhibition of *Curtobacterium* sp. #2 was rescued by the presence of *A. laibachii*, suggesting a protective effect of *A. laibachii* on *Curtobacterium* sp. #2. An interesting observation was made regarding the transcriptional induction of MbA_gh25. In our previous study (23), we had initially identified gh25 as a candidate gene based on its transcriptional activation on the plant surface specifically in the presence of *A. laibachii*. Here, we find that gh25 expression in MbA was significantly upregulated in the presence of both *A. laibachii* and *Curtobacterium* sp. #2. However, also confrontation of MbA with *Curtobacterium* sp. #2, *Curtobacterium* sp. #1, and *Pseudomonas* sp. #1 alone resulted in transcriptional activation of gh25. In contrast, antibiotic-treated *A. laibachii* alone did not induce gh25 expression in MbA (Fig. 5D

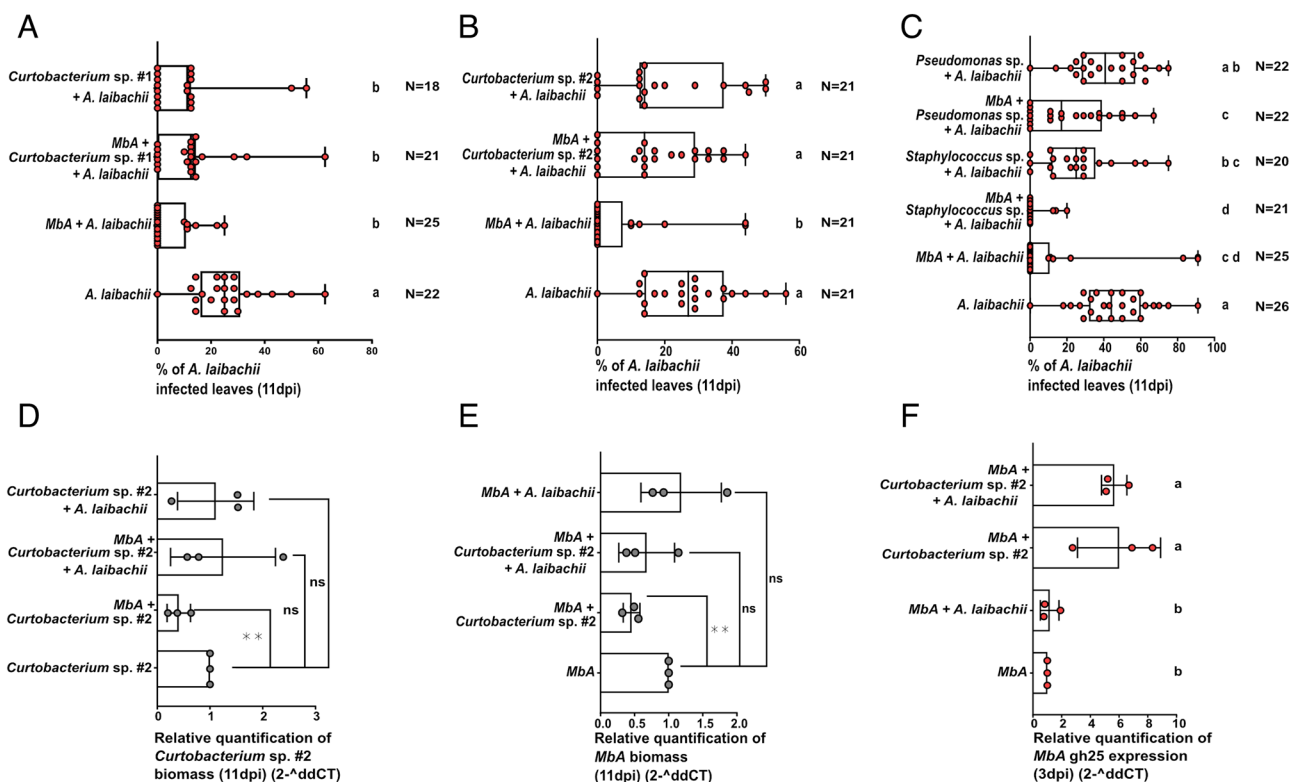


Fig. 5. *Curtobacterium* sp. #2 suppresses MbA inhibition of *A. laibachii*. (A–C) Infection assay of *A. laibachii* on *A. thaliana* with pretreatment of MbA and different bacteria; (A) *Curtobacterium* sp. #1, (B) *Curtobacterium* sp. #2, (C) *Staphylococcus* sp. and *Pseudomonas* sp. pretreatment was performed 2 d prior to *A. laibachii* infection. Plants were scored at 11 dpi. Kruskal–Wallis and Dunn’s multiple comparisons test (alpha 0.05) was performed to find significant difference between treatments. (D) Relative quantification of *Curtobacterium* sp. #2 biomass in response to *A. laibachii* and MbA. 23S rRNA specific region in *Curtobacterium* sp. was normalized to *A. thaliana* EF1- α to quantify the amount of *Curtobacterium* DNA in the samples at 11 dpi. (E) Relative quantification of MbA biomass in response to *A. laibachii* and *Curtobacterium* sp. GH25 (g2490) as a single-copy gene was normalized to *A. thaliana* EF1- α to quantify the amount of MbA DNA in the samples at 11 dpi. Unpaired t test carried out to find significant difference between treatments. Both *Curtobacterium* sp. #2 and MbA biomass was significantly reduced upon interaction with one another. (F) Relative quantification of MbA gh25 expression in response to *A. laibachii* and *Curtobacterium* sp. #2, normalized to MbA housekeeping gene *ppi* at 3 dpi. One-way ANOVA and Tukey’s multiple comparisons test (alpha 0.05) was performed to find significant difference between treatments.

and *SI Appendix, Fig. S8*). Following up, we tested *gh25* expression upon coinoculation with nontarget bacteria *Microbacterium* sp. 2 and *Chryseobacterium* sp., which did not lead to *gh25* expression (*SI Appendix, Fig. S8*). From these results we concluded that not *A. laibachii* itself, but its associated bacteria, mainly *Curtobacterium* sp., trigger *gh25* expression in MbA.

Next, we investigated whether the observed strain-specific inhibitory activity of GH25 was restricted to MbA_GH25 or might be a conserved property of GH25 proteins across clades. To test this, we performed confrontation assays with the *FB1* strains overexpressing GH25 orthologs derived from MbA, *U. maydis* or *R. solani* (Fig. 2A) and *Curtobacterium* sp. #2. Consistent with GH25 antagonism of *A. laibachii* infection, GH25 from MbA and *U. maydis* inhibited *Curtobacterium* sp. #2 (*SI Appendix, Fig. S9A*), while GH25 from *R. solani* had no effect on *Curtobacterium* sp. #2. In a control experiment, phylogenetically unrelated bacterial strain (*Priestia* sp.) was inhibited by enzymatically active versions of all three GH25 orthologs (*SI Appendix, Fig. S9B*). This result suggests an ortholog-dependent target specificity of GH25, which may reflect a host-specific adaptation.

Finally, we tested whether the protective effect of *Curtobacterium* sp. #2 on *A. laibachii* can be attributed to its modulation of the leaf microbiome. We examined potential cross-inhibition within the microbial community by spreading one bacterium as a lawn and applying treatment bacteria to observe any resulting halo formation (*SI Appendix, Fig. S10*). This showed that *Curtobacterium* sp. #1 and #2, as well as *Pseudomonas* sp. #1 and

#2, inhibited most of the associated bacteria that were not targeted by GH25 (Fig. 6A). Furthermore, when these bacteria were used as a lawn, the inhibited bacteria were unable to grow, suggesting strong inhibitory potential (*SI Appendix, Fig. S10A*). Based on these findings, we hypothesized that *Curtobacterium* sp. and *Pseudomonas* sp. might promote *A. laibachii* growth through their inhibitory effects on other microbes. To test this, we conducted an infection assay using *Microbacterium* sp. #2 and *Chryseobacterium* sp., two bacteria that are not inhibited by GH25 but are targeted by both *Pseudomonas* sp. and *Curtobacterium* sp. We found that both *Microbacterium* sp. #2 and *Chryseobacterium* sp. displayed an inhibitory effect on *A. laibachii* and were unable to alleviate inhibition of *A. laibachii* by MbA (Fig. 6B). In addition, they rescued the growth inhibition of *A. thaliana* caused by *A. laibachii* (*SI Appendix, Fig. S10B*). To further support our hypothesis, we tested whether this effect could be reversed by coinoculating *Curtobacterium* sp. #2 and *Pseudomonas* sp. #1, respectively. *Curtobacterium* sp. #2 could restore *A. laibachii* infection in the presence of both *Microbacterium* sp. #2 and *Chryseobacterium* sp., *Pseudomonas* sp. in the presence of *Chryseobacterium* sp. (Fig. 6C). Taken together, our findings reveal a tripartite, interkingdom interaction between bacteria, oomycete, and yeast, in which a GH25 lysozyme shapes a highly specific antagonism between MbA, *Curtobacterium* sp., and *A. laibachii*. We propose that this interplay between organisms on the leaf enables a balanced competition and maintenance of microbial niches in the plant phyllosphere (Fig. 7).

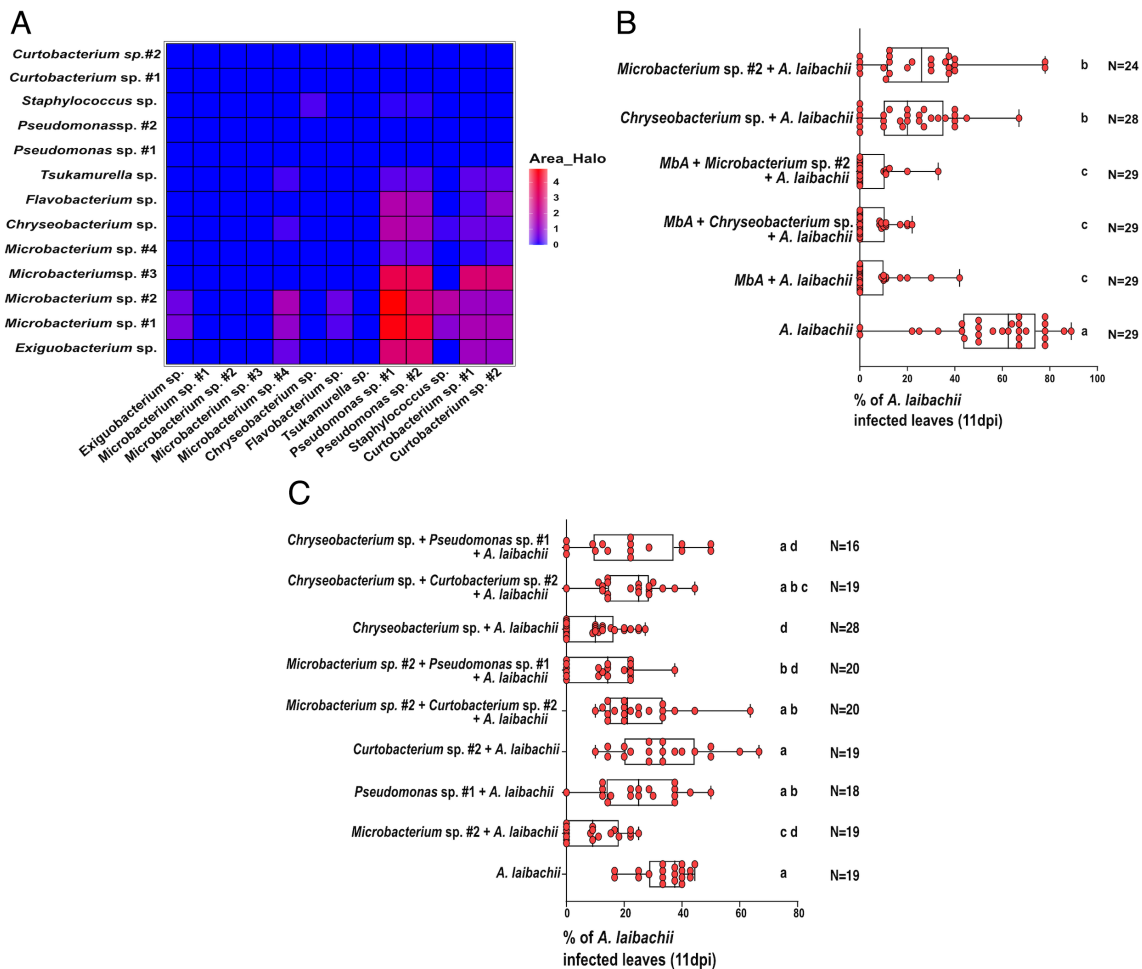


Fig. 6. Events within the associated bacterial community shape infection outcome. (A) Inhibition assays between bacteria were conducted on square PD plates. 250 μ L lawn bacterium was plated out and 5 μ L competitor/treatment bacterium spotted on top. After 3 to 4 d halo area was measured and analyzed using ImageJ. 3 to 4 biological replicates were performed for each bacterial pair tested. Mean of replicates is used for plotting. (B) Infection assay of *A. laibachii* on *A. thaliana* with pretreatment of MbA and *Microbacterium* sp. #2 and *Chryseobacterium* sp. Pretreatment was performed 2 d prior to *A. laibachii* infection. Plants were scored at 11 dpi. Kruskal-Wallis and Dunn's multiple comparisons test (alpha 0.05) was performed to find significant difference between treatments. (C) Infection assay of *A. laibachii* on *A. thaliana* with pretreatment of *Microbacterium* sp. #2, *Chryseobacterium* sp., *Pseudomonas* sp. #1, and *Curtobacterium* sp. #2. Kruskal-Wallis and Dunn's multiple comparisons test (alpha 0.05) was performed to find significant difference between treatments.

Discussion

We previously described how the basidiomycete yeast MbA antagonizes the oomycete plant pathogen *A. laibachii* through a GH25 lysozyme secreted by MbA (23). Here, we demonstrate that the observed antagonism between MbA and *A. laibachii* is mediated through an *A. laibachii* associated *Curtobacterium* sp. in a strain-specific manner.

We tested two different modes of antagonism, including a direct action of GH25 on the oomycete cell wall, as well as an indirect inhibition of infection via the activation of plant immune responses. We could not obtain experimental evidence for either of these modes. Simultaneously, we found that MbA and GH25 were unable to antagonize oomycete pathogens *Hpa* and *P. infestans*. However, lack of evidence for *P. infestans* inhibition may also be based on a potential inability of MbA/U. maydis to grow on *N. benthamiana* leaves, which has not been investigated so far. Although both *A. laibachii* and *Hpa* are foliar pathogens in natural *A. thaliana* populations, *Hpa* is less abundant and more affected by perturbations in key hormonal pathways in the host (30). For instance, while *A. laibachii* growth is unaffected in abscisic acid (ABA) and salicylic acid (SA) mutant lines of *A. thaliana*, *Hpa* growth is restricted on jasmonic acid (JA) accumulating ABA mutant line and increased on sid2-2 mutant, defective in

Isochorismate Synthase 1 (precursor in SA signaling) (30). Nevertheless, *A. candida* is known to suppress broad spectrum host innate immunity and increase susceptibility of the plant to downy mildew infection (31, 32).

Several reports have shown *Hpa* disease symptoms to be controlled by the presence of bacteria. Berendsen et al. (33) reported three bacterial taxa (*Stenotrophomonas* sp., *Xanthomonas* sp., and *Microbacterium* sp. to become enriched in the *A. thaliana* rhizosphere upon infection with *Hpa*. Moreover, in a study by Almario et al. (2022), although *Hpa* comprised the core taxa, the sampled leaves were asymptomatic due to the presence of plant beneficial bacterium such as *Sphingomonas* and *Variovorax* (33, 34). Additionally, it was demonstrated that the *Hpa*-associated microbiota in *A. thaliana* inhibits the further sporulation of the oomycete (29). Therefore, the presence of *Hpa* on the host plant is highly affected by associated microbiota. Similarly, for *P. infestans*, fungal endophytes isolated from different host plants inhibited *P. infestans* during coculture conditions (35). Since both *Hpa* and *A. laibachii* are obligate biotrophic pathogens, their survival depends on the living host and therefore maintaining a stable equilibrium in the host-microbial community is crucial (30, 36).

Exploring microbiota associated with pathogens can offer insights into disease emergence and proliferation (37). For

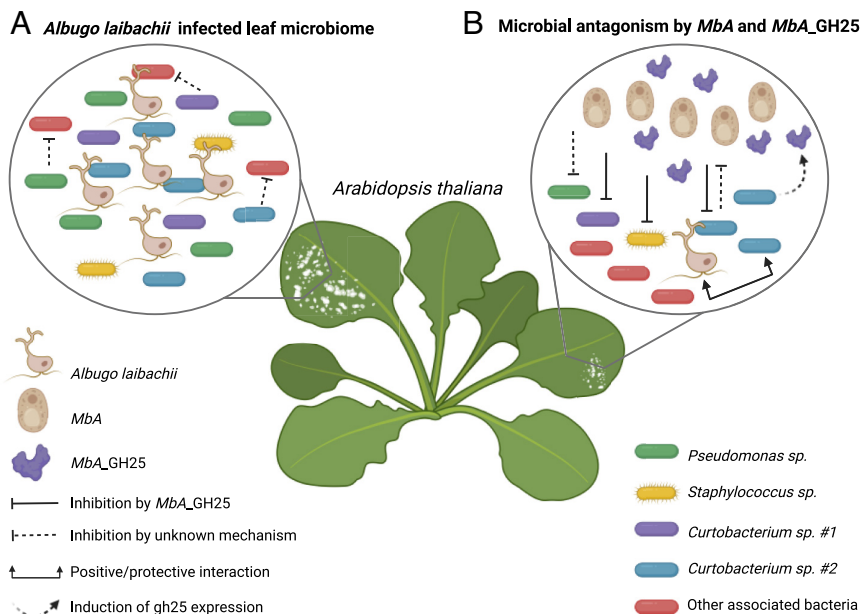


Fig. 7. Model of the interkingdom interactions shaped by *MbAGH25*. We depict how white rust infection in *A. thaliana*, caused by oomycete *A. laibachii* is inhibited by basidiomycete yeast *MbA* through antagonism of oomycete associated bacteria. (A) *A. laibachii* is closely associated with a bacterial consortium in the phyllosphere of *A. thaliana*. Particularly, *Curtobacterium* sp. and *Staphylococcus* sp. are positively correlated with the host infection caused by *A. laibachii*. (B) Basidiomycete yeast *MbA* produces GH25 protein to target *Curtobacterium* sp. #2, which leaves *A. laibachii* unprotected from competition with *MbA*, leading to a reduction of white rust infection in *A. thaliana*. Created in <https://BioRender.com>.

example, *A. laibachii* infections help *P. infestans* to colonize and sporulate on *Arabidopsis* (38), which could result from *Albugo* infections in Brassicaceae promoting a host jump by certain pathogens (39). *Albugo candida* has been shown to directly influence the plant microbiome by releasing proteins and peptides with antimicrobial activity into the apoplast (40). Moreover, microbiome analysis during the interaction between *A. thaliana* and *A. laibachii* revealed the formation of a disease associated microbial community (designated as “DCom”) in infected plants, distinct from the health-associated microbial community (designated as “HCom”) found in uninfected controls (10). Furthermore, coinoculation with HCom members more effectively suppressed *A. laibachii* infection than coinoculation with DCom-associated microbes (10).

Therefore, we were intrigued to explore how associated bacteria of *A. laibachii* might be a key component in the *MbA*_GH25 mediated inhibition of *A. laibachii*. To this end, we isolated 14 bacterial members from *A. laibachii* Nc14 spores and tested for interaction with *MbA* and *MbA*_GH25. During the confrontation assays, we found the *Pseudomonas* sp. strains to be inhibited by *MbA*, and not *MbA*_GH25, which indicates that the antimicrobial activity against *Pseudomonas* is independent of GH25 secretion. The *MbA* induced bacterial inhibition could be potentially caused by biosurfactants or secondary metabolites as described previously for related yeasts (41). Interestingly, two *Curtobacterium* sp. isolates were inhibited by GH25, with *Curtobacterium* sp. #2 survivability being strongly impaired. However, only *Curtobacterium* sp. #2 rescued the inhibition of *A. laibachii* by *MbA*, reflecting a high level of specificity. Moreover, both *Curtobacterium* sp. #2 and *MbA* significantly antagonize each other *in-planta*, indicating a bidirectional negative interaction. Finally, *gh25* gene expression in *MbA* was upregulated in the presence of *Curtobacterium* sp. #2 rather than *A. laibachii*. Furthermore, we found *Curtobacterium* sp. and *Pseudomonas* sp. to be key microbes in targeting most of the *A. laibachii* isolated associated bacteria that are not inhibited by GH25, and rescuing

A. laibachii infection also in the presence of antagonistic bacteria such as *Chryseobacterium* sp. Taken together, our observations reveal a tripartite antagonism between *MbA*, *Curtobacterium* sp. #2, and *A. laibachii*, on *A. thaliana* phyllosphere. In conjunction with results that demonstrate a preference of *A. laibachii* toward DCom microbes (10) and the general role of *A. laibachii* as a hub microbe in shaping the microbial community (42) one could hypothesize that the association with *Curtobacterium* sp. #2 aids *A. laibachii* in maintaining the stability of its niche. Unlike *Hpa*, which is associated with bacteria inhibiting the pathogens infection (29), *A. laibachii* thus might have developed mechanisms to maintain a microbiome including protective microbes.

Simultaneously, we tested GH25 orthologs from *U. maydis* and *R. solani* in the antagonism toward *A. laibachii*. We found that GH25 from closely related basidiomycetes *MbA* and *U. maydis* were able to inhibit both *A. laibachii* *in-planta* and *Curtobacterium* sp. #2 during *in vitro* confrontation assays, as opposed to that from phylogenetically more distant plant pathogen *R. solani*. Therefore, these results indicate a host-specific adaptation of the three GH25 fungal orthologs. Further investigation of GH25 evolution and functional diversification in the fungal kingdom is a tempting objective for future studies.

It has been shown that filamentous plant pathogens secrete effectors to modulate the plant microbiota (42–44). For example, the secretion of phytotoxic ribonucleases by fungal pathogens *Zymoseptoria tritici* (45) and *U. maydis* (46) can modify the leaf microbiome of their respective hosts. Regarding GH25 specificity, one could hypothesize that adaptation to competing bacteria in the leaf phyllosphere contributes to the niche adaptation of pathogenic fungi and increases the fitness of beneficial fungi, such as *MbA*, to establish an epiphytic lifestyle in association with the highly competitive *A. laibachii*.

Future research should focus on understanding the mechanisms that determine the positive interaction between *A. laibachii* and *Curtobacterium* sp. #2 as well as elucidating genetic differences and variances in the cell wall composition between the two

Curtobacterium sp. strains. Understanding how pathogens select for bacteria enhancing their pathogenicity might pave the way for discovery of new biologicals interrupting these interactions. Moreover, validating the observed interactions in more complex microbial communities would be intriguing.

In conclusion, we propose a model depicting the cross-kingdom interaction between bacteria, oomycete, and yeast orchestrated by GH25 (Fig. 7). Our study reveals a tripartite interkingdom interaction between the yeast *MbA*, the oomycete *A. laibachii*, and the bacterium *Curtobacterium* sp. #2 on the plant surface. We show that *MbA* and *Curtobacterium* sp. #2 exhibit mutual antagonism, with *A. laibachii* providing a protective effect on *Curtobacterium* sp. #2 and vice versa. The presented data further suggest a host-specific adaptation of GH25 orthologs in microbial competition and direct targeting of specific bacteria leading to a reshaping of the microbial community structure. Therefore, understanding the underlying mechanisms of competition between plant pathogens and associated microbes is crucial for effective disease control and restoration of plant health.

Materials and Methods

Growth Conditions for Microbial Strains. *MbA* and *U. maydis* strains were grown in liquid YEPS light medium at 22 °C and 28 °C respectively in a rotary shaker (200 rpm) and maintained on potato dextrose (PD) agar plates. *A. laibachii* associated bacterial strains (Dataset S2) were grown in King's B liquid media at 22 °C overnight in a rotary shaker (200 rpm). *Pichia pastoris* KM71H-OCH was used for recombinant protein expression as described by ref. 23.

Plant Infection Assays. *A. laibachii* infections in *A. thaliana* were adapted from the protocol described by ref. 23. Experimental set-up in Fig. 1 A and B followed exact instructions described by ref. 23 to reproduce published data, whereby for all other experiments the procedure has been optimized, scoring at 11 dpi instead of 14 dpi. *A. laibachii* infections of the ortholog experiment were performed in nonsterile conditions. Fresh shoot weight was taken at 11 dpi and roots were removed for the measurements. Detailed protocols for *Hpa Noco2* (47), *P. infestans* strain 88069 and *B. cinerea* B05.10 infection assays can be found in SI Appendix.

Isolation of *A. laibachii* Associated Bacteria. *A. laibachii* spore suspension was prepared as described in ref. 30 (SI Appendix). After antibiotic treatment of the spores, spores were diluted 1:10, 1:100, and 1:1,000 and 50 µL of each dilution and the undiluted suspension were plated on King's B media (self-made). Plates were incubated at room temperature (20 to 22 °C) and after 3 to 5 d single colonies were picked. The identity of the isolated strains was determined via partial 16S ribosomal RNA (rRNA) sequencing using 16S rRNA primer pairs (SI Appendix for PCR conditions and purification and Dataset S1). Sequences were blasted to the NCBI database and best hit listed if >97% identity. See Dataset S2 for information on the isolated bacteria and the respective sequences.

Microbial Confrontation Assays. For bacterial confrontation assays, *MbA/U. maydis* cultures grown to OD_{600nm} 1.0 were dropped (10 µL) in four quadrants of a PD agar plate, spread previously with 100 µL of bacterial culture with an OD_{600nm} of 0.6. 20 µL of GH25 protein (0.8 µg/µL) was applied to a hole created in the center of the plate (d = 4 mm). For bacterial cross-inhibition assays, square PD plates were used. Bacteria were diluted to an OD₆₀₀ of 0.8 in 10 mM MgCl₂ and 250 µL of a lawn bacterium was spread. 5 µL of competitor bacteria were dropped on the plate. Plates were incubated for 2 to 4 d at 22 °C. See SI Appendix for *P. infestans* and *B. cinerea* confrontation assays.

Cell Viability Assays. Two different assays were performed to access cell viability after GH25 treatment: a colony-forming units assay (SI Appendix) and luciferase assay using the BacTiter-GloTM Microbial Cell Viability Assay (Promega, Madison, USA). For the luciferase assay, the following four strains were tested: *Curtobacterium* sp. #1, *Curtobacterium* sp. #2, *Staphylococcus* sp., and *Pseudomonas* sp. #1. Each strain was grown overnight and then diluted to an OD₆₀₀ of 0.1 in 10 mM phosphate buffer in 500 µL reactions. Active and mutated (D124A) GH25 was added to a final concentration of 10 µM. A buffer-only control was included. The reaction was incubated for 1 h at 37 °C. Protocol was performed according to the manufacturer's instructions and relative survival was

calculated by setting the buffer control to 100% and using the following formula: ("RLU_treated"/"RLU_buffer_control")*100.

Bacterial Lysis Assay. To assess the lytic activity of lysozyme, the two isolated *Curtobacterium* sp. strains were incubated with either a commercial lysozyme (hen egg white lysozyme, HEWL, from the EnzChek® Lysozyme Assay Kit, Thermo Fisher) or purified active and inactive GH25 (SI Appendix for detailed protocol).

Elicitor Assays. Protocol was modified from Cao et al. (2014) (48). See SI Appendix for more information.

Nucleic Acid Methods. Total RNA extraction from plant samples were performed with Trizol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions followed by treatment with the Turbo DNA-Free Kit (Ambion/Applied Biosystems; Darmstadt, Germany) to remove any DNA contamination in the extracted RNA. Synthesis of complementary DNA (cDNA) was performed using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Darmstadt, Germany) according to recommended instruction starting with a concentration of 10 µg RNA. RT-qPCR oligonucleotide pairs were designed with Primer3 Plus. Relative expression levels of marker genes were analyzed with GoTaq® qPCR Master Mix in a Bio-Rad iCycler system using the following program: 2 min at 95 °C followed by 45 cycles of 30 s at 95 °C, 30 s at 61 °C and 30 s 72 °C. Plasmid Prep Kit (QIAGEN, Venlo, The Netherlands) was used for isolation of plasmid DNA from bacteria after the principle of alkaline lysis. Genomic DNA was isolated using phenol-chloroform extraction protocol (30).

Construction of Plasmids. All plasmids were constructed using either restriction enzyme mediated ligation using T4 DNA ligase or Gibson assembly (New England Biolabs; Frankfurt a.M., Germany). All GH25 ortholog gene expression constructs (Dataset S1) were constructed using Gibson assembly. *R. solani* GH25 was codon optimized for expression in *U. maydis* and synthesized gene was obtained from BioCat GmbH, Heidelberg. GH25 active site mutants created by using the QuickChange XL Site-Directed Mutagenesis Kit from Agilent Technology according to the manufacturer's instructions. *E. coli* transformation was performed using heat shock according to standard molecular biology methods (49). Vector constructs and oligonucleotide sequences are mentioned in Dataset S1. Generated constructs were verified at the Eurofins sequencing facility (Germany).

Fungal Transformation and Validation. The heterologous gene expression constructs (Dataset S1) were introduced in SG200 or FB1 (GH25 ortholog overexpression) by ipi integration, using protoplasts according to Kämper (2004) (50). Generated strains were further analyzed by southern blot to validate the integration and copy number. Expression was validated by RNA extraction of growing axenic culture followed by qRT-PCR (Nucleic Acid Methods).

Phylogenetic Analysis. For phylogenetic tree construction, all selected GH25 orthologs (see ref. 23) as well as all bacterial 16S rRNA sequences were aligned using MAFFT Multiple Sequence Alignment Software Version 7. Subsequently, a phylogenetic tree was constructed using Neighbor-Joining with default settings and 100 bootstrap replications using Phyl.io (51).

Statistical Analysis. Graphpad Prism (10.5.0) was used to generate data plots. For statistical analysis, we first checked for normal distribution of datasets using the Shapiro-Wilk test. For normally distributed datasets, ordinary one-way or two-way ANOVA and Tukey's multiple comparisons test was conducted to find significant differences between groups. For datasets lacking normal distribution, Kruskal-Wallis and Dunn's multiple comparisons test was conducted to find significant differences between groups. ImageJ 1.53K version (Wayne Rasband and Contributors NIH, USA) was used to calculate necrotic lesions of *P. infestans*, *B. cinerea*, and zone of inhibition by fungal and yeast strains. Fiji (2.16.0) was used to analyze the area of inhibition zones in bacterial confrontation assay. Di-Codon Optimization of *R. solani* GH25 was performed using Python 3.x (Python Software Foundation, 2023).

Data, Materials, and Software Availability. 16S sequencing data are shown in Dataset S2. All microbial strains are available on request from the corresponding authors. All other data are included in the article and/or supporting information.

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