The Epiphytic Fungus *Pseudozyma aphidis* Induces Jasmonic Acid- and Salicylic Acid/Nonexpressor of PR1-Independent Local and Systemic Resistance$^{1[C][W]}$

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*Pseudozyma* spp. are yeast-like fungi, classified in the Ustilaginales, which are mostly epiphytic or saprophytic and are not pathogenic to plants. Several *Pseudozyma* species have been reported to exhibit biological activity against powdery mildews. However, previous studies have reported that *Pseudozyma aphidis*, which can colonize plant surfaces, is not associated with the collapse of powdery mildew colonies. In this report, we describe a novel *P. aphidis* strain and study its interactions with its plant host and the plant pathogen *Botrytis cinerea*. This isolate was found to secrete extracellular metabolites that inhibit various fungal pathogens in vitro and significantly reduce *B. cinerea* infection in vivo. Moreover, *P. aphidis* sensitized Arabidopsis (*Arabidopsis thaliana*) plants’ defense machinery via local and systemic induction of *PATHOGENESIS-RELATED1* (PR1) and *PLANT DEFENSIN1.2* (PDF1.2) expression. *P. aphidis* also reduced *B. cinerea* infection, locally and systemically, in Arabidopsis mutants impaired in jasmonic acid (JA) or salicylic acid (SA) signaling. Thus, in addition to direct inhibition, *P. aphidis* may inhibit *B. cinerea* infection via induced resistance in a manner independent of SA, JA, and Nonexpressor of PR1 (NPR1). *P. aphidis* primed the plant defense machinery and induced stronger activation of *PDF1.2* after *B. cinerea* infection. Finally, *P. aphidis* fully or partially reconstituted *PR1* and *PDF1.2* expression in *npr1-1* mutant and in plants with the SA hydroxylase *NahG* transgene, but not in a *jasmonate resistant1-1* mutant, after *B. cinerea* infection, suggesting that *P. aphidis* can bypass the SA/NPR1, but not JA, pathway to activate PR genes. Thus, either partial gene activation is sufficient to induce resistance, or the resistance is not directed solely through PR1 and PDF1.2 but probably through other pathogen-resistance genes or pathways as well.

Plants encounter a vast array of pathogens during their growth and development, including viruses, bacteria, and fungi. To cope with constant attacks by invading pathogens, plants have evolved a wide range of defense mechanisms, including the use of preexisting physical and chemical barriers. Pathogens that pass these first two layers of defense may encounter the hypersensitive response—the rapid induction of localized cell death—that prevents their spread beyond local infection (Glazebrook, 2005). Those pathogens that persist and overcome the hypersensitive response still need to challenge the well-orchestrated plant defense responses, which include systemic acquired resistance (SAR; Perfect et al., 1999) or induced systemic resistance (Heil and Bostock, 2002; Somssich, 2003; Haas and Défago, 2005; Grant and Lamb, 2006).

Plant-defense responses that are activated in response to pathogen attack are regulated by different communicating signaling pathways in which signaling molecules such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play major roles (Dong, 2004; Durrant and Dong, 2004; Beckers and Spoel, 2006; Broekaert et al., 2006; Loake and Grant, 2007). The SA-dependent pathway correlates with the local and SAR response, especially against biotrophic pathogens. This usually results in the induced expression of SAR-marker pathogenesis-related (PR) genes (e.g. PR1, PR2, PR5, and BGL2-encoding genes) in local and systemic plant tissues (van Loon et al., 2006). PR gene induction can be either dependent on the NONEXPRESSOR OF PR1/NONINDUCIBLE IMMUNITY1 (NPR1/NIM1) protein or NPR1 independent (Dong, 2004). In the NPR1-dependent pathway, NPR1 “senses” the SA signal through changes in redox status in the plant cell, and low redox potential can lead to translocation of NPR1 into the cell nucleus (Kinkema et al., 2000; Mou et al., 2003). In the nucleus, NPR1 regulates the expression of PR proteins through interactions with the transcription factor TGA (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000; Fan and Dong, 2002; Wang et al., 2005). Cytosolic NPR1 is also involved in the cross talk between the SA- and JA-signaling pathways and acts as a negative regulator in the synergistic effect of SA on the JA response (Spoel et al., 2003). The defense-signaling pathway that is mediated by JA and ET plays an important role in plant defense responses against necrotrophic...
pathogens and herbivorous insects (Penninckx et al., 1996; Penninckx et al., 1998; Thomma et al., 1998).

Fungal biocontrol agents have become an important alternative to the use of chemicals because of environmental concerns. Biological control can be achieved by one or a combination of mechanisms, including antibiosis, mycoparasitism, competition, and induced resistance in the host plant. These mechanisms may hinder pathogen growth and development, thereby reducing disease. The complex mode of action of biocontrol agents reduces the likelihood that pathogens will develop a resistance to them (Elad and Freeman, 2002; Shoresh et al., 2010).

Studies indicate that biocontrol agents can protect plants from diverse pathogens by inducing systemic resistance mechanisms that are often associated with up-regulation of PR genes and/or with accumulation of phytoalexins (Shoresh et al., 2005; Hossain et al., 2007; Stein et al., 2008; Shoresh et al., 2010). Trichoderma asperellum, for example, is an effective biocontrol agent for a number of soilborne pathogens of cucumber (Cucumis sativus). T. asperellum infection modulates the expression of genes involved in the JA/ET-signaling pathways of inducing systemic resistance (Shoresh et al., 2005). Similarly, in Arabidopsis (Arabidopsis thaliana), resistance to powdery mildew (Golovinomyces cichoracearum) can be conferred by the mycorrhizal fungus Piriformospora indica through systemic resistance (Stein et al., 2008). P. indica induces resistance via JA signaling and NPR1 (Stein et al., 2008), and Penicillium simplicissimum can induce resistance in Arabidopsis by activating multiple defense mechanisms, including both SA- and JA/ET-signaling pathways (Hossain et al., 2007). Transcription analysis of plant interaction with Trichoderma hamatum failed to detect induction of induced systemic resistance markers, and only one marker of SAR (PR5) was up-regulated (Alfano et al., 2007). Therefore, it is likely that different biocontrol agents use different mechanisms to induce plant defense.

Epiphytic yeasts colonizing different plant surfaces (Last and Price, 1969; Phaff and Starmer, 1987; Pusey et al., 2009; Fernández et al., 2012) are thought to have biocontrol activity and to provide a natural barrier against some plant pathogens (Fokkema et al., 1975; Fokkema and Schippers, 1986; Starmer et al., 1987; Avis and Bélanger, 2001; Urquhart and Punja, 2002; Bleve et al., 2006; Jacobsen, 2006; Robiglio et al., 2011). Raacke et al. (2006) demonstrated that yeasts can activate systemic acquired resistance in Arabidopsis plants and reduce symptoms of Botrytis cinerea infection in an SA-dependent, but JA-independent, manner. In contrast, symptoms of Pseudomonas syringae infection were reduced independently of the JA or SA pathways (Raacke et al., 2006).

The genus Pseudozyma is a small group of Basidiomycota related to the Ustilaginales (Boekhout, 1995). These fungi are mostly epiphytic or saprophytic and are not pathogenic to plants or animals (Avis and Bélanger, 2002). Pseudozyma rugulosa and Pseudozyma flocculosa have both been found to exhibit biological activity against the different powdery mildews with which they are associated (Jarvis et al., 1989; Hajlaoui and Bélanger, 1991, 1993; Bélanger et al., 1994; Hajlaoui et al., 1994; Dik et al., 1998; Hammami et al., 2011). P. flocculosa has been found to secrete an unusual fatty acid that displayed antibiotic activity against several pathogens (Hajlaoui et al., 1994; Benyagoub et al., 1996; Avis and Bélanger, 2001; Avis et al., 2001). Studies on other Pseudozyma species (e.g. Pseudozyma antarctica and Pseudozyma prolifici) have suggested that those species hold great potential for biocontrol applications and production of novel fatty acids that may be useful for cosmetics and other industries (Avis and Bélanger, 2001). P. aphidis is a close relative of P. rugulosa (Begerow et al., 2000), which was first isolated from aphid secrections (Henninger and Windisch, 1975), but was later also identified on plant surfaces (Allen et al., 2004). Avis et al. (2001) reported that P. aphidis isolated from aphid se- cretions (isolate CBS 517.83) is not associated with col- ony collapse of powdery mildew, and that it does not produce unique antifungal fatty acids. More recent studies, however, have shown that P. aphidis is a novel producer of mannosylethritol lipids, and while the possible function of these lipids as antifungal agents needs to be determined, their value as a surfactant in the cosmetic industry has been recognized (Rau et al., 2005).

In our laboratory, we isolated a unique strain of P. aphidis (designated isolate L12; Levy and Gafni, 2011). Here we demonstrate that the epiphytic yeast P. aphidis isolated from the surface of strawberry (Fragaria × ananassa) leaves (isolate L12) has biocontrol ability against fungal phytopathogens with a dual mode of action: antibiosis and induced resistance that is independent of JA and SA/NPR1.

RESULTS
Isolation of a Unique P. aphidis Strain

We isolated a strain of P. aphidis (isolate L12) from strawberry leaves. The L12 isolate was classified as P. aphidis using specific primers for the entire ribosomal DNA region of the internal transcribed spacer and for the partial sequence of the mitochondrial large subunit and nuclear small subunit as described by Avis et al. (2001). Sequences showed 100% identity to P. aphidis (Supplemental Fig. S1; Supplemental Materials and Methods S1).

The fungus secreted pinkish metabolites when grown on potato (Solanum tuberosum) dextrose agar (PDA; Fig. 1A). Light and scanning electron microscopy (SEM) revealed that P. aphidis isolate L12 is a dimorphic epiphytic fungus. We demonstrated that the fungus can have a yeast-like form (Fig. 1, B and E) and synnema-like structure (Fig. 1C) on PDA, and can also form hyphae (Fig. 1, D and I). The fungus could also grow on and cover both host (tomato [Solanum lycopersicum]) and model (Arabidopsis) leaf surfaces (Fig. 1, F and G).

Foliar application of P. aphidis at two different concentrations (10^4 and 10^6 spores mL^{-1}) on tomato and Arabidopsis plants and detached leaves showed no
evidence of pathogenicity or symptoms of plant sensitivity up to 4 weeks post application. Similarly, there was no evidence of pathology associated with tomato plants after drenching the root system with a suspension of the biocontrol agent.

\( \text{P. aphidis} \) Secretions Inhibit Fungal Pathogens in Vitro

\( \text{P. aphidis} \) L12 isolate secretes extracellular pinkish-colored metabolites (Fig. 1A). We tested the growth-inhibitory effect of \( \text{P. aphidis} \) extracts on \( \text{B. cinerea} \), which causes gray mold, and \( \text{Alternaria brassicicola} \), which causes dark leaf spot in most Brassica species, by measuring growth haloes around filters saturated with hexane extracts of \( \text{P. aphidis} \) L12 isolate culture-filtrate metabolites. We obtained inhibition haloes of 13 mm for \( \text{B. cinerea} \) and 19 mm for \( \text{A. brassicicola} \) with \( \text{P. aphidis} \) secretion extracts and no inhibition with extracts from potato dextrose broth (PDB) only as a control (Fig. 2A). We next revealed that PDA containing \( \text{P. aphidis} \) secretions can completely inhibit the spore germination of \( \text{B. cinerea} \) and \( \text{A. brassicicola} \) (Fig. 2B), as well as their mycelium linear growth (Supplemental Fig. S2, A and B). Inhibition of \( \text{B. cinerea} \) and \( \text{A. brassicicola} \) mycelium linear growth persisted even when autoclaved \( \text{P. aphidis} \) secretions were used (Supplemental Fig. S2C). Since \( \text{P. aphidis} \) secretions could inhibit \( \text{B. cinerea} \) in vitro, we further examined its ability to inhibit pathogen symptoms on plants.

\( \text{P. aphidis} \) Inhibits \( \text{B. cinerea} \) Infection on Tomato Plants

Detached tomato leaves were sprayed with \( \text{P. aphidis} \) \((10^4 \text{ or } 10^8 \text{ spores mL}^{-1})\) 3 d before inoculation with \( \text{B. cinerea} \). Infection was significantly reduced, by 70% to 80% and by almost 100%, when leaves were sprayed with \( 10^4 \) or \( 10^8 \text{ spores mL}^{-1} \) of \( \text{P. aphidis} \), respectively, as compared with leaves sprayed with water (control; Fig. 3, A and B). Application of \( 10^8 \text{ P. aphidis} \) spores mL\(^{-1}\) on

![Figure 1](image1.png)

**Figure 1.** \( \text{P. aphidis} \) growth on PDA plate and on plants. A, \( \text{P. aphidis} \) after 10-d growth on PDA media; arrow indicates secretions. B, Yeast-like growth shape on PDA (light microscopy from above). C, Synnema-like appearance on PDA (light microscopy, profile). D, \( \text{P. aphidis} \) mycelium/yeast-like form on PDA (SEM, profile). E, \( \text{P. aphidis} \) yeast-like form on PDA (SEM, from above). F, \( \text{P. aphidis} \) after 3-d growth on Arabidopsis leaf (SEM). G, \( \text{P. aphidis} \) mycelium-like form on tomato (light microscopy, profile). H, Spore shape on PDB. I, Spore shape on yeast-malt-peptone-dextrose (YMPD) liquid medium (hemicytometer, light microscopy).

![Figure 2](image2.png)

**Figure 2.** In vitro inhibition of fungal phytopathogens by \( \text{P. aphidis} \) secretions. A, Extracts of \( \text{P. aphidis} \) culture filtrate fraction (PA) or PDB fraction (Control) were used for fungal-inhibition halo assay. B, PDA (Control) and PDA containing \( \text{P. aphidis} \) secreted fraction (PA) were used for spore germination-inhibition assays. Percentage germination inhibition by PA relative to control is shown. [See online article for color version of this figure.]
intact tomato plants in the greenhouse 3 d before inoculation with *B. cinerea* also reduced infection of *B. cinerea* by 60% to 75% (Fig. 3C). Autoclaving *P. aphidis* abolished the inhibitory effects on *B. cinerea* infection (Fig. 3C). The ability of live *P. aphidis* to inhibit *B. cinerea* symptoms on plants could be complex, involving antibiosis combined with some other direct mode of action such as competition or parasitism, or an indirect mode of action such as induced resistance. To verify what other modes of action, if any, are used by *P. aphidis*, we further monitored *P. aphidis*-*B. cinerea* interactions on the plant and the ability of *P. aphidis* to induce resistance.

**P. aphidis-B. cinerea Interaction in Planta**

The direct interaction of *P. aphidis* with *B. cinerea* was studied on Arabidopsis leaves by SEM 24 h after inoculation with *B. cinerea*. We observed a higher proliferation or accumulation of *P. aphidis* in the lesion area (Fig. 4, A and D), forming many more hyphae than yeast-like morphs in the interaction area (Fig. 4B), as compared with the uninfected area (Fig. 4C). *P. aphidis* cells seemed to adhere to the *B. cinerea* hyphae and spores (Fig. 4B).

**P. aphidis** Application Activates Plant-Induced Resistance and Priming

We studied the effect of *P. aphidis* on the activation of the plant defense responses in three Arabidopsis mutants. When Arabidopsis mutants impaired in JA signaling (*jasmonate resistant1-1* [jar1-1]; Staswick et al., 1992) and in SA accumulation and signaling (the salicylate hydroxylase-expressing transgenic plant NahG; Gaffney et al., 1993), and *npr1-1* (Cao et al., 1994), were treated with *P. aphidis* and then inoculated with *B. cinerea*, local disease inhibition was as strong as in wild-type plants (Fig. 5, A and B). By applying *P. aphidis* to only one of the Arabidopsis rosette leaves for 24 h and then inoculating the other leaves with *B. cinerea*, disease inhibition in systemic leaves was demonstrated in the wild type as well as in NahG, *npr1-1*, and jar1-1 mutants (Fig. 5C). PR1 expression was up-regulated 4,000-fold, and *PLANT DEFENSE SINE1.2* (*PDF1.2*) expression was up-regulated 2.5-fold relative to that in untreated wild-type Arabidopsis plants 3 d after application of the live culture of *P. aphidis*, but not after application of autoclaved culture (Fig. 6A). While induction of *PR1* in NahG and *npr1-1* plants was not significantly induced 3 d post treatment with *P. aphidis*, it was up-regulated in the jar1-1 mutant, albeit 1,000-fold less than in the *P. aphidis*-treated wild type (Fig. 6A). *PDF1.2* expression, which was not as markedly affected as *PR1* in the wild type, was similarly induced in NahG and *npr1-1* plants, but it was not up-regulated at all in the jar1-1 mutants (Fig. 6A). *PR1* and *PDF1.2* gene up-regulation was demonstrated locally and systemically in wild-type plants as early as 24 h after treatment with *P. aphidis* (Supplemental Fig. S3). *PDF1.2* gene expression was induced 7.5-fold both locally and systemically; *PR1* expression was systemically induced 21-fold, but only 2.5-fold locally as compared with the untreated wild type (Supplemental Fig. S3). When gene expression was monitored in *P. aphidis*-treated versus untreated plants 24 h post infection with *B. cinerea*, we observed up-regulation of *PDF1.2* and *PR1* by 17-fold and 72-fold, respectively, in *P. aphidis*-treated versus untreated wild-type plants (Fig. 6B),
while the expression of PR1 was 60-fold less than that observed in *P. aphidis*-treated uninfected wild-type plants (Fig. 6A). In *npr1-1* plants, the expression of both genes was only partially recovered; in *NahG* plants, *PDF1.2* expression was similar to that in the wild type whereas *PR1* expression was partially reconstituted, and in *jar1-1* plants neither gene was expressed after infection (Fig. 6B).

**DISCUSSION**

In this article we examined the ability of the epiphytic fungus *P. aphidis* (isolate L12) to control *B. cinerea* disease and explored its mode of action. We demonstrated *P. aphidis* colonization and establishment on plant surfaces (Figs. 1 and 4) as previously described (Allen et al., 2004). The protective effect of *P. aphidis* against plant pathogens was demonstrated by both direct antagonistic interactions and induced resistance. In contrast to Avis et al. (2001), likely due to the use of different isolates, the *P. aphidis* isolate used in this work secreted metabolites that have inhibitory effects on several phytopathogenic fungi in vitro (Fig. 2; Supplemental Fig. S2) and also inhibit gray mold and other plant diseases in planta (Fig. 3; A. Gafni, I. Rahat, and M. Levy, unpublished data). Secreted metabolites inhibited both spore and linear hyphal growth of fungal pathogens in vitro (Fig. 2; Supplemental Fig. S2). Application of *P. aphidis* before pathogen inoculation significantly reduced the gray mold-causing agent *B. cinerea* on tomato plants in the greenhouse, and disease inhibition was abolished when *P. aphidis* was not vital (Fig. 3). The application of a biocontrol agent before plants are exposed to a pathogen can sometimes improve biocontrol activity (Filonow et al., 1996), as demonstrated in this study. However, in other studies, no significant effects were observed (Chalutz and Wilson, 1990; Cook et al., 1997).

Metabolite secretion was not the only mechanism of *P. aphidis* biocontrol; we also observed local and systemic activation of inducible defense mechanisms in plants treated with *P. aphidis* (Fig. 5). Activation of inducible mechanisms in plants is potentially suitable for the enhancement of plant resistance to pathogens (Shoresh et al., 2010). We found that spraying Arabidopsis plants with live culture of *P. aphidis* leads to activation of PR genes from the SA and JA pathways (Fig. 6A; Supplemental Fig. S3), indicating that *P. aphidis* mediates induced resistance via both pathways. Further analysis with Arabidopsis SA-pathway mutants *NahG* and *npr1-1* and JA pathway mutant *jar1-1* showed that *P. aphidis* can reduce *B. cinerea* infection locally but also systemically in Arabidopsis mutants impaired in JA or SA signaling, i.e. *jar-1-1*, *NahG*, and *npr1-1* (Fig. 5C). The data suggest that beyond the direct local antagonistic inhibition, *P. aphidis* inhibits *B. cinerea* infection systemically via induced resistance in a SA/NPR1- and JA-independent manner. Defensive resistance can be locally induced at the site of inoculation or systemically throughout the whole plant; our results with hormonal-defective mutants suggest that both modes are activated by *P. aphidis*.

*B. cinerea* activated *PDF1.2* expression 17-fold and *PR1* expression 72-fold in plants treated with *P. aphidis* versus untreated plants (Fig. 6B). However, *PR1* expression was actually antagonized by *B. cinerea*, while
PDF1.2 was enhanced as compared with uninfected plants treated with *P. aphidis* (Fig. 6A). A proposed scenario in wild-type plants, which has also been suggested by Tucci et al. (2011) for *Trichoderma* spp., might be that in the absence of pathogen, preventative treatment with *P. aphidis* activates expression of PR genes from both SA and JA pathways, in order to prime the plant defense reaction. However, when these treated plants are inoculated with *B. cinerea*, the JA-dependent response is enhanced as a consequence of the priming effect, eventually resulting in the observed increase in local and systemic resistance. Since we found that *P. aphidis* activates induced resistance in a JA- and SA-independent manner, it was reasonable to suggest that this antagonistic effect is connected to different or unknown defense pathways, and not to the known antagonistic effect between SA and JA pathways. We further found that *P. aphidis* treatment could partially or fully recover *PR1* and *PDF1.2* expression in *npr1-1* and *NahG* mutants but not in *jar1-1* mutants (Fig. 6). This suggests that *P. aphidis* can bypass the SA/NPR1 pathway, but not the JA pathway, to activate pathogenesis genes. Thus, either partial gene activation is sufficient to induce resistance, or the resistance is not directed solely through *PR1* and *PDF1.2* but also through other putative pathogen-resistance genes and/or pathways. This suggests that other hormone-independent pathways or PR genes are also involved in the resistance induced by *P. aphidis* or, perhaps as yet unknown, partially SA/NPR1- and JA-dependent pathways. The combination of hormone-dependent and-independent pathways as a mode of action for induced resistance has been demonstrated in plants treated with *Trichoderma* spp. or yeast (Raacke et al., 2006; Korolev et al., 2008).
Resistance to *B. cinerea* can result from a combination of several mechanisms, including plant response, antifungal metabolite secretion (as indicated in our in vitro experiments; Fig. 2), and attachment of *P. aphidis* to *B. cinerea* spores, preventing their germination (as indicated by our SEM observations; Fig. 4; Allen et al., 2004). Another direct effect could be delayed spore germination due to decreased hydrophobicity of the leaf surface. The abundance of *P. aphidis* in *B. cinerea*-infected tissue and the close relationship between the two organisms (Fig. 4) might suggest parasitism, although we could not demonstrate this in vitro (Supplemental Fig. S4); however, it might also suggest competition for nutrients because conidial germination in many *B. cinerea* isolates is nutrient dependent and *P. aphidis* outcompeting *B. cinerea* conidia for available nutrients would reduce disease severity (Bashi and Fokkema, 1977; Fokkema et al., 1979; Dik et al., 1991, 1992; Filonow et al., 1996; Barnett et al., 2000).

Although activation of an inducible defense mechanism in plants is potentially sufficient to enhance plant resistance against pathogens, it should be noted that different pathogens induce different, mostly complex sets of responses, including accumulation of JA, SA, and secondary metabolites, and sometimes these responses differ only in their kinetics or magnitude, which are crucial for the outcome of the interactions. Furthermore, external factors (e.g. plant genotype, environmental conditions, etc.) can easily increase this complexity, also influencing the outcome.

In conclusion, our results demonstrate that *P. aphidis* has the potential for use as a biocontrol agent of fungal pathogens. The mechanisms of action involved in *P. aphidis* control are antifungal metabolite secretion and induced resistance, which are independent of or partially dependent on the JA and SA/NPR1 pathways. However, to increase our knowledge of *P. aphidis* mechanisms of action, it would be useful to further characterize the interaction with the plant and determine whether there are other as yet unknown pathways involved in the activation of the observed induced resistance.

RNA Isolation and Quantitative Reverse Transcription PCR Analysis

Total RNA was isolated from untreated Arabidopsis plants and from plants 24 to 72 h post treatment with 10^8 *P. aphidis* spores mL^-1 withQiagen RNeasy kit (Invitrogen) according to the manufacturer’s instructions or using the LogSpin method (Yaffe et al., 2012). DNase treatment was performed on Qiagen columns according to the manufacturer’s instructions (Invitrogen). Total RNA (1 μg) was reverse transcribed with EZ-First strand cDNA synthesis kit (Biological Industries). Quantitative reverse transcription PCR was performed with the SYBR master mix and StepOne real-time PCR machine (Applied Biosystems). The thermal cycling program was as follows: 95°C for 20 s and 40 cycles of 95°C for 3 s and 60°C for 30 s. Relative fold change in gene expression normalized to Aipt Shibif in samples from inoculated versus uninoculated Arabidopsis leaves was calculated by the comparative cycle threshold method. The primer sequences were as follows: Aipt TB1F, 5'-GACTGTGAATGTTAAGGCTTTAGCG-3'; Aipt TB1R, 5'-GCGTTAGATCAGGAAAGTGTATAGTCTCTG-3'; Czeckowski et al., 2005); Aipt PR1F, 5'-GGCTTGAGATGTTAAGGCTTTAGCG-3'; Aipt PR1R, 5'-GTTTGGGACATCCGATGTCT-3'; AiptDF1-2F, 5'-TCTGCTTGTCTCCTTGCTC-3'; AiptDF1-2R, 5'-GTGTCGAGGAGACATA-3' (Conn et al., 2008).

Isolation of *P. aphidis*-Secreted Fraction for in Vitro Inhibition Assays

Secreted metabolites were extracted from PDB or PDB culture filtrate of *P. aphidis* using ethyl acetate followed by hexane. We grew *P. aphidis* in PDB medium at 26°C for 10 d in Erlenmeyer flasks at a constant agitation of 150 rpm. We then spun down the fungal cells (20 min at 10,000 rpm). The supernatant, consisting of culture filtrate, was titrated to pH 2.0 using 1 N HCl and extracted with an equivalent volume of ethyl acetate using separating funnels. The ethyl-acetate fraction was collected, reextracted with hexane, and evaporated in a rotor evaporator (Buchi) at 42°C (Paz et al., 2007). The dry fraction was reconstituted in methanol and used for in vitro experiments after application on Whatman paper discs (6 mm diameter). The discs were placed in the center of the PDA plates embedded with the pathogen spores.

In Vitro Inhibition Assays using *P. aphidis*-Secreted Fraction

*P. aphidis* was placed on PDA covered with dialysis tubing and incubated at 26°C for 10 d. We then removed the tubing with the fungi and used the plates with the *P. aphidis*-secreted fraction for inhibition assays with different fungal pathogens. Alternatively, we grew *P. aphidis* directly on PDA and used the bottom side of the PDA for inhibition assays. We inoculated control PDA plates and PDA plates containing the *P. aphidis* secretions with the different pathogens, incubated them at their optimum temperatures, and estimated their spore germination or hyphal growth.

Inhibition of *B. cinerea* on Tomato Plants

To examine inhibition of *B. cinerea* on detached tomato leaves and whole plants, we sprayed the leaves/plants with different concentrations (10^4 and 10^6 spores mL^-1) of *P. aphidis* to flowing, allowed it to become established for 3 d on the leaves/plants, inoculated them with the pathogen (total of 7,500 spores per leaflet), and monitored disease symptoms relative to plants sprayed with water.

Electron Microscopy

SEM of *P. aphidis*-B. cinerea interactions was performed using a standard protocol (Weigel and Glazebrook, 2002). Arabidopsis leaves were sprayed with *P. aphidis* and 3 d later were inoculated with *B. cinerea*. After 24 h, leaf samples were fixed with glutaraldehyde, critical-point dried (BAL-TEC), mounted on aluminum stubs, sputter coated with gold, and examined by SEM at an accelerating voltage of 20 kV.

Determination of Local and Induced Resistance

We monitored PR gene expression levels in Arabidopsis plants 3 d after foliar application of live culture of *P. aphidis* (10^8 cells mL^-1) relative to plants

### MATERIALS AND METHODS

**Pseudozyma aphidis Culture**

*Pseudozyma aphidis* isolate L12 was maintained in solid culture on PDA (Difco) at 26°C and transferred to fresh medium monthly. Liquid cultures were maintained in PDB (Difco) for 7 to 10 d at 26°C on a rotary shaker at 150 rpm. We obtained 10^8 cells mL^-1 after 5 to 10 d in liquid culture.

**Propagation of Plants and Pathogens**

*Botrytis cinerea* (B05.10) and *Alternaria brassicicola* were grown on PDA medium at 22°C to 27°C under 12-h daily illumination.

*Tomato (Solanum lycopersicum '870')* plants were grown at 25°C and 40% relative humidity in the greenhouse. Arabidopsis (*Arabidopsis thaliana*) accession Columbia and its mutants jir1-1, NahG, and npr1-1 were used. All seeds were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). All Arabidopsis seeds were vernalized on moist soil at 4°C for 2 to 3 d before placing them in a growth chamber. Arabidopsis plants were grown at 22°C and 60% relative humidity under illumination with fluorescent and incandescent light at a photosynthetic rate of approximately 120 μmol m^-2 s^-1 day length was 12 h unless otherwise specified.
treated with water (control). We also monitored gene expression 24 h after infection with B. cinerea of untreated and P. aphidicola-treated plants. Plants showing local resistance were inoculated with B. cinerea 3 d post P. aphidicola application, and lesion size was monitored 72 h post infection. For systemic resistance to B. cinerea, lesion formation was assayed 3 d after infection in systemic leaves. In this experiment, infection was performed 24 h post application of P. aphidicola to the bottom leaf and not 72 h post application as in the first experiment, so as to avoid spreading of P. aphidicola to the upper leaves as is known to happen after 24 h.

Statistical Analysis

Student’s t tests were performed only when data were normally distributed and the sample variances were equal. Otherwise Mann-Whitney U tests were performed only when data were normally distributed. The following tests were performed: The Student’s t test was performed. Significance was accepted at P < 0.05 and is noted in the text or figure captions. All experiments shown here are representative of at least three independent experiments with the same pattern of results.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Identification of L12 isolate.

Supplemental Figure S2. In vitro inhibition of fungal phytopathogens by P. aphidicola secretions.

Supplemental Figure S3. Local and systemic induced resistance by P. aphidicola.

Supplemental Figure S4. In vitro parasitism assay.

Supplemental Materials and Methods S1.

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LITERATURE CITED


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