Pseudozyma aphidis Induces Salicylic-Acid-Independent Resistance to *Clavibacter michiganensis* in Tomato Plants

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Abstract

Barda, O., Shalev, O., Alster, S., Buxdorf, K., Gafni, A., and Levy, M. 2015. *Pseudozyma aphidis* induces salicylic-acid-independent resistance to *Clavibacter michiganensis* in tomato plants. *Plant Dis.* 99:621-626.

The ability of plant pathogens to rapidly develop resistance to commonly used pesticides challenges efforts to maximize crop production. Fungal biocontrol agents have become an important alternative to chemical fungicides as a result of environmental concerns regarding conventional pesticides, including resistance issues. The complex mode of action of biocontrol agents reduces the likelihood that pathogens will develop resistance to them. We recently isolated a unique, biologically active isolate of the epiphytic fungus *Pseudozyma aphidis*. We show that the extracellular metabolites secreted by our *P. aphidis* isolate can inhibit *Xanthomonas campestris* pv. *vesicatoria*, *X. campestris* pv. *campestris*, *Pseudomonas syringae* pv. *tomato*,

Erwinia amylovora, *Clavibacter michiganensis*, and *Agrobacterium tumefaciens* in vitro. Moreover, application of *Pseudozyma aphidis* spores on tomato plants in the greenhouse significantly reduced (by 60%) the incidence of bacterial wilt and canker disease caused by *C. michiganensis* subsp. *michiganensis* on those plants as well as disease severity by 35%. Furthermore, infected plants treated with *P. aphidis* were 25% taller than control infected plants. We found that *P. aphidis* activates *PR1a*—and other pathogenesis-related genes in tomato plants—and can trigger an induced-resistance response against *C. michiganensis* that proceeds in a salicylic-acid-independent manner, as shown using NahG-transgenic tomato plants.

Plant pathogens can rapidly develop resistance to pesticides, which can result in immense annual losses in yield. Fungal biocontrol agents have become an important alternative to the use of chemicals due to environmental concerns, including resistance issues. Repeated use of the same class of pesticides to control a pathogen can increase the proportion of less susceptible individuals in the pathogen population. In contrast, the ability of pathogens to develop resistance to biological control agents, which have complex modes of action (e.g., antibiosis, mycoparasitism, competition, or induced resistance in the host plant), is limited.

One important mode of action used by biocontrol agents to help protect plants from pathogens is induction of the plant's systemic resistance mechanisms. This induction process is often associated with upregulation of pathogenesis-related (PR) genes or accumulation of phytoalexins (37,59,60,63). Studies of different fungal biocontrol agents have demonstrated the effectiveness of some of them against a variety of plant pathogens. For example, *Trichoderma asperellum* was shown to activate induced systemic resistance by modulating the expression of genes involved in the jasmonic acid/ethylene (JA/ET)-signaling pathways. This mechanism provided effective biocontrol of a number of soilborne pathogens of cucumber (60). *Penicillium simplicissimum* was also found to activate an induced resistance response in *Arabidopsis* that involves JA/ET-signaling but also salicylic acid (SA)-signaling pathways (37). A similar effect was shown for the mycorrhizal fungus *Piriformospora indica*, which conferred resistance to powdery mildew through systemic resistance via JA signaling and NPR1 (63). On the other hand, transcriptional analysis of the interaction of *T. hamatum* with tomato plants failed to

detect any induction of JA markers and, in the context of that interaction, only one systemic acquired resistance marker (PR5) was upregulated (2). These findings suggested that different mechanisms and pathways are used by different biocontrol agents to induce plant defense responses.

Biocontrol activity and natural barriers against certain plant pathogens can be provided by epiphytic yeasts colonizing different plant surfaces (4,27,28,42,51,62,66). Diseases in the greenhouse and in storage have been shown to be controlled by specific yeasts and yeast-like fungal species and strains (15,20,21,23,24,26,40,46,48,55). The genus *Pseudozyma* is a small group of yeast-like fungi that are related to the order Ustilaginales (11). *Pseudozyma* spp. are non-pathogenic to plants and animals and mostly epiphytic or saprophytic (4). Some species of *Pseudozyma*, such as *Pseudozyma rugulosa* and *P. flocculosa*, exhibit biological activity against different powdery mildews (9,22,33–35,39). One possible mode of action in *P. flocculosa* is secretion of an unusual fatty acid that exhibits antibiotic activity against several pathogens (3,5,10,33). On the other hand, Avis et al. (5) showed that *P. aphidis* isolate CBS 517.83 does not secrete any antifungal fatty acids, nor does it inhibit *Podosphaera xanthii* (formerly *Sphaerotheca fuliginea*), the causal agent of powdery mildew. In our previous study (12,13), we demonstrated that *P. aphidis* isolate L12, isolated from the surface of strawberry leaves, can serve as a biocontrol agent against *Botrytis cinerea* on *Arabidopsis* plants and that its biocontrol activity involves a dual mode of action: induced resistance and antibiosis. In that work, we concluded that the induced resistance observed in the *Arabidopsis* plants was JA, SA, and ET independent and that *P. aphidis* can bypass the SA/NPR1 pathway but not the JA pathway in order to activate the PR genes *PR1* and *PDF1.2* (12,13).

In the current work, we demonstrate the potential of *P. aphidis* isolate L12 for use as a biocontrol agent against bacterial pathogens. We found that the secreted fraction of *P. aphidis* can inhibit a variety of plant bacterial pathogens in vitro and activate induced resistance against the bacterial pathogen *Clavibacter michiganensis* in tomato plants in an SA-independent manner.

Materials and Methods

***P. aphidis* culture.** The *P. aphidis* isolate L12 was grown on potato dextrose agar (PDA; Difco) at 26°C and transferred to fresh medium monthly. Liquid cultures in potato dextrose broth (PDB; Difco)

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures are published online.

Accepted for publication 11 October 2014.

were inoculated with two 1-cm² blocks of PDA carrying mycelia or spores. The liquid culture was grown for 7 to 10 days on a rotary shaker set at 150 rpm and kept at 26°C. We obtained a concentration of 10⁸ conidia/ml after 10 days of growth in liquid culture. *P. aphidis* concentrations were determined by counting the cells in a hemocytometer. For long-term storage, *P. aphidis* spores were kept in 50% glycerol at -80°C.

RNA isolation and quantitative real-time polymerase chain reaction analysis. Total RNA was isolated from tomato plants (*Solanum lycopersicum* cultivar 870) sprayed with water (control) and from treated plants 24 to 72 h after treatment with *P. aphidis* at 10⁸ spores/ml (suspended in water), with or without infection with *C. michiganensis*. This isolation was carried out using the Qiagen RNeasy kit (Invitrogen, San Diego, CA), according to the manufacturer's instructions, or the LogSpin method (72). DNase treatment was performed using Qiagen columns, according to the manufacturer's instructions (Invitrogen). Total RNA (1 µg) was reverse-transcribed with an EZ-First-Strand cDNA synthesis kit (Biological Industries, Israel). Quantitative real-time polymerase chain reaction (PCR) was performed with SYBR master mix on a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA). 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 *Tip41* expression in samples from treated versus water-sprayed (control) tomato leaves was calculated using the 2^{-ΔΔC_t} method (44). The primer sequences are listed in Table 1 (7,25).

Isolation of *P. aphidis*-secreted fraction for in vitro inhibition assays. Secreted metabolites were extracted from PDB culture filtrate using ethyl acetate and hexane. One and a half liters of PDB medium in a 3-liter Erlenmeyer flask was inoculated with two 1-cm² blocks of PDA carrying mycelia or spores of *P. aphidis* and grown for 21 days, kept at 26°C with constant agitation at 150 rpm. We then spun down the fungal cells (20 min at 10,000 rpm). The supernatant, consisting of culture filtrate, or PDB only 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, Flawil, Switzerland) at 42°C (49). The dry fraction was reconstituted in methanol and used for in vitro experiments after the application of an equivalent of 2 mg of the dry fraction to each Whatman paper disc (6 mm in diameter). The discs were placed in the center of PDA plates, which were inoculated with the different bacteria. Bacterial suspension (4 ml, 10⁹ cells/ml) were embedded in each 200 ml of PDA (cooled to 45 to 50°C) before pouring into petri dishes. The

inhibition halo was measured after 48 h at the optimal temperature (depicted in parenthesis below) that was used for the pathogen's propagation.

Propagation of plants and pathogens. *C. michiganensis* subsp. *michiganensis* strain CMM44 (30°C), *Xanthomonas campestris* pv. *vesicatoria* (28°C), *X. campestris* pv. *campestris* (28°C), *Agrobacterium tumefaciens* (32°C), *Erwinia amylovora* (37°C), and *Pseudomonas syringae* pv. *tomato* (27°C) (from our private collection) were grown on nutrient agar medium (NA; Difco) in a controlled incubator in complete darkness at the indicated temperatures.

Tomato plants (*S. lycopersicum* 870) were grown in soil in a greenhouse kept at a constant temperature of 25°C and 40% relative humidity under natural light conditions. Plants were kept in plastic containers which were filled with water for irrigation. The mutant tomato line *NahG* (29,47) and plants expressing its wild-type background (ecotype 'Money Maker') were also grown under the same conditions.

Inhibition of *C. michiganensis* infection of tomato plants. Two-week-old greenhouse-grown tomato plants (*S. lycopersicum* cultivar 870) planted in pots (15 cm in diameter) filled with soil mixture were sprayed with water (control) or with *Pseudozyma aphidis* (10⁸ spores/ml suspended in water) until the spore suspension flowed off the plant. *P. aphidis* was allowed 2 to 3 days to become established on the plants. Then, the plants were inoculated with *C. michiganensis* subsp. *michiganensis* strain CMM44 that had been grown in nutrient broth at 30°C at a constant agitation of 150 rpm, by cutting the first leaf with scissors that had been dipped in the bacterial suspension (optical density at 600 nm of approximately 0.9). Plants from the different treatments were randomized in the greenhouse. Twenty-eight days after this inoculation, we rated the disease symptoms on these plants and on plants that had been treated only with water. Disease incidence was expressed as the percentage of plants that developed symptoms. Disease severity was assessed on a 0-to-5 scale as follows: 0 = 0% of leaves showing wilting; 0.5 = slight marginal wilting, 10% of leaves with wilt; 1 = 20% of leaves with wilt; 1.5 = 30% of leaves with wilt; 2 = 40% of leaves with wilt; 2.5 = 50% of leaves with wilt; 3 = sectorized wilting, 60% of leaves showing wilting associated with chlorosis; 3.5 = 70% of leaves with wilt; 4 = 80% of leaves with wilt; 4.5 = pronounced leaf collapse, 90% of leaves showing wilting; and 5 = 100% of plant is wilted. A mean disease severity index was calculated for each treatment by adding up the scores of the 10 to 15 plants in the treatment and then expressing that sum as a percentage using the formula described by Raupach et al. (57): disease severity index = [Σ(rating number × number of plants in rating) × 100%]/(total number of plants × highest rating). Biocontrol efficacy was calculated using the formula described here: efficacy = [(disease rate in control - disease rate in treatment)/disease rate in control] × 100.

Determination of defense activation. The expression of PR genes was monitored in tomato plants 3 days after foliar application of *P. aphidis* (10⁸ spores/ml, suspended in water) and compared with expression in plants treated with water (control) and with autoclaved dead *P. aphidis*. Gene expression in untreated and *P. aphidis*-treated plants was also monitored after inoculation with *C. michiganensis*.

Statistical analysis. Student's *t* tests were performed only when data were normally distributed and the sample variances were equal. In all other cases, the Tukey-Kramer honestly significant difference test was used. Significance was accepted at *P* < 0.05 (as noted in the text or table captions). All experiments described here are representative of at least two independent experiments with the same pattern of results.

Results

Extracts of *P. aphidis* secretions inhibit a range of bacterial pathogens in vitro. The growth-inhibitory effect of *P. aphidis* extracts on several bacteria was evaluated by measuring the halos around filter paper saturated with extracts of *P. aphidis* isolate L12

Table 1. Primer sequences for pathogenesis-related genes used in this study

Primer name	Sequence 3'-5'
SITIP41 For	GCTGCGTTTCTGGCTTAGG
SITIP41 Rev	ATGGAGTTTGTAGTCTTCTGC
SIPR1a For	GTCTGGCGCAACTCAGTCCGA
SIPR1a Rev	TGCAAGAAATGAACCACCACCCA
SILoxD For	GCAACGAGGACTGGTGGCCC
SILoxD Rev	TTGCGCTGAAGCCAGCCAGA
SILePIN2 For	AATTATCCATCATGGCTGTTCAC
SILePIN2 Rev	CCTTTTGGATCAGATTCTCCTT
SILeChi3-For	TGCAGGAACATTCACTGGAG
SILeChi3-Rev	TAACGTTGTGGCATGATGGT
SILeChi9-For	GAAATTGCTGCTTTCCTTGC
SILeChi9-Rev	CTCCAATGGCTCTTCCACAT
SIGlucA-For	GGTCTCAACGCGACATATT
SIGlucA-Rev	CACAAGGGCATCGAAAAGAT
SIGlucB-For	TCTTGCCCCATTCAAGTTC
SIGlucB-Rev	TGCACGTGTATCCCTCAAAA
SIPTI5 For	GAGAGTATGGCTAGGTACGTTCG
SIPTI5 Rev	TAAGTAGTGCCTTAGCACCCTCGC
SIPR5 For	ATCGAGGCCAAACATGGGTCTAT
SIPR5 Rev	TTGGCATGAACCTCTACCAGCA

culture-filtrate metabolites. The inhibition of several bacterial pathogens' growth was observed (Fig. 1). A strong inhibitory effect of the extracts on *C. michiganensis* subsp. *michiganensis* (4.3-cm² halo), which causes bacterial wilt and canker in tomato plants, as well as on *X. campestris* pv. *vesicatoria* (1.7-cm² halo), the causal agent of bacterial leaf spot on pepper and tomato, was observed. The extracts also had moderate inhibitory effects (0.7- to 1.2-cm² halo) on other bacterial pathogens: *A. tumefaciens*, *E. amylovora*, *Pseudomonas syringae* pv. *tomato*, and *X. campestris* pv. *campestris* (Fig. 1). No inhibitory effect was found on any of the pathogens using extracts from PDB only as control (data not shown).

***P. aphidis* reduces *C. michiganensis* infection in tomato.** Application of suspensions of *Pseudozyma aphidis* spores (10⁸ spores/ml) to tomato plants in the greenhouse 3 days prior to inoculation with *C. michiganensis* provided 60% control of bacterial wilt and canker disease 38 days after inoculation (Fig. 2). At this time point, we observed 100% mortality of the untreated plants whereas only 40% of the plants that had been treated with *P. aphidis* were dead (Fig. 2). At 28 days after inoculation, disease severity among sick plants that had been treated with *P. aphidis* was significantly lower (by 34.6%) than that observed among the water-treated (control) plants (Table 2), and inoculated *P. aphidis*-treated plants were 25.3% taller than the inoculated control plants (Table 2). In addition, at that time point, there was a difference between the distribution of disease severity in the two treatments. Although all of the untreated plants showed symptoms of disease, 22% of the plants that had been treated with *P. aphidis* were completely free of disease symptoms (Fig. 3). Among the untreated plants, 27% exhibited disease symptoms (but remained alive) and 73% were dead. In comparison, 47% of the *P. aphidis*-treated plants exhibited disease symptoms (but remained alive) and only 31% were dead (Fig. 3).

***P. aphidis* induces resistance in tomato plants.** Three days after the application of live *P. aphidis* culture, expression of the PR genes *PR1a* and *PR5*, chitinases 9 and 3 (*Chit9* and *Chit3*, respectively), glucanases A and B (*GlucA* and *GlucB*, respectively), and PTO-interacting protein 5 (*PTI5*) were upregulated relative to their expression levels in the untreated wild-type plants. However, no significant activation of the PR genes lipoxygenase D (*LOX*) and proteinase inhibitor II (*PIN2*) was observed in either treatment (Fig. 4A; Supplementary Fig. S1A). *PR1a* was upregulated 30-fold relative to its expression in the water-treated wild-type plants and it

was the gene most upregulated by *P. aphidis*; therefore, we used it as our marker gene in subsequent experiments. In plants that had been inoculated with *C. michiganensis*, *PR1a* expression was upregulated 10- to 11-fold before symptoms appeared, as compared with the uninfect, water-treated control plants. However, in plants that were treated with *P. aphidis* before being inoculated with *C. michiganensis*, *PR1a* was synergistically upregulated (29- to 30-fold) as compared with the control plants (Fig. 4B). Other PR genes were also upregulated after inoculation with *C. michiganensis* but no further activation was seen when plants were pretreated with *P. aphidis*. *PR1a* was not activated when we applied dead autoclaved *P. aphidis* on plants (Fig. 4B).

***P. aphidis* inhibits *C. michiganensis* on tomato plants in an SA-independent manner.** When transgenic tomato plants with impaired SA accumulation (the salicylate hydroxylase-expressing transgenic plant [*NahG*]) (29,47) were treated with *P. aphidis* and then inoculated with *C. michiganensis*, disease severity was similar to that observed among the wild-type plants (Table 3). This finding demonstrates that *P. aphidis* inhibits *C. michiganensis* in an SA-independent manner. We also found that, in *NahG* plants, the expression of *GlucB* was significantly upregulated 3 days after treatment with *P. aphidis*, while *PR1a* and other PR genes demonstrated no activation or minor upregulation (Supplementary Fig. S2).

Discussion

We recently isolated a unique, biologically active isolate of *P. aphidis* from strawberry leaves and demonstrated that this isolate can reduce *B. cinerea* infection of tomato and *Arabidopsis* plants (12,13). We further demonstrated that this biocontrol effect involves a dual mode of action (antibiosis and induced resistance) that is SA/NPR1 and ET/JA independent (12,13).

In the current work, we examined this isolate's ability to control bacterial plant pathogens. The *P. aphidis* isolate secreted metabolites that have inhibitory effects on several phytopathogenic bacteria in vitro. The secreted metabolites have not yet been identified but we demonstrated that crude extracts of these metabolites can inhibit several different bacterial pathogens in vitro (Fig. 1), including *C. michiganensis*, *E. amylovora*, *Pseudomonas syringae* pv. *tomato*, and *X. campestris* pv. *vesicatoria*. Antibiotic secretion is well established as a common mode of action of biocontrol agents (6,32,43,56). We demonstrated that *P. aphidis* has an inhibitory effect on *C. michiganensis* in planta (Fig. 2; Table 2), in contrast to Avis

Fig. 1. In vitro inhibition of phytopathogens by *Pseudozyma aphidis* secretions. Crude hexane extract of *P. aphidis* secretions (2 mg) was used for each bacterial inhibition assay. Whatman paper discs (6 mm in diameter) saturated with crude extract was placed on a potato dextrose agar plate containing the pathogen and inhibition halos were measured 48 h later. XCV = *Xanthomonas campestris* pv. *vesicatoria*, XCC = *X. campestris* pv. *campestris*, PST = *Pseudomonas syringae* pv. *tomato*, EA = *Erwinia amylovora*, CM = *Clavibacter michiganensis*, and AT = *Agrobacterium tumefaciens*. Results represent means $\pm$ standard error ($n = 6$). Shown here are the results of one experiment that was representative of at least four experiments with similar results.

Fig. 2. In vivo inhibition of *Clavibacter michiganensis* by *Pseudozyma aphidis*. Whole 2-week-old greenhouse-grown tomato plants were sprayed with *P. aphidis* spores (10⁸ spores/ml; PA) or water (control) 3 days before being inoculated with *C. michiganensis* (optical density at 600 nm of approximately 0.9). Disease symptoms were monitored for 38 days following the inoculation and dead plants were scored. Asterisk denotes significant differences as determined using a Student's *t* test, $P < 0.05$. Results represent percentage of dead plants per treatment ($n = 15$). Shown here are the results of one experiment that was representative of at least four experiments with similar results.

et al. (5), who demonstrated that *P. aphidis* provides no biocontrol; the difference between their findings and ours is most likely due to the use of different isolates (5). We demonstrated that application of *P. aphidis* before pathogen inoculation significantly reduces (by 60%) the incidence of bacterial wilt and canker infection of tomato plants caused by *C. michiganensis* in the greenhouse (Fig. 2); moreover, disease severity was also reduced, by 34.6%. Furthermore, *P. aphidis*-treated plants that were infected with *C. michiganensis* were 25.3% taller than infected, water-treated plants (Table 2). This might be explained by the fact that the *P. aphidis*-treated plants are healthier but also suggests as-yet-uncharacterized production or modulation of plant hormones, as demonstrated previously in plants treated with other beneficial microbes (30).

C. michiganensis subsp. *michiganensis* is the causal agent of the wilt and canker disease that is the most destructive bacterial disease in tomato plants worldwide (18). These bacteria enter into the host plant via wounds and natural openings and colonize the plant's vascular tissue. Systemic colonization of xylem vessels leads to leaf wilting, leaflet necrosis, and the spread of cankers on the plant stems, a process that eventually concludes in plant death (38). *C. michiganensis* infection activates the tomato plant's ET-dependent basal defense responses (7).

Application of *P. aphidis* to tomato plants (cultivar 870) activates their SA- and ET-dependent resistance pathways, as demonstrated by the activation of the *PR1a* and *PTI5* marker genes (16,64) (Fig. 4). However, it does not affect the expression of the *LOX* gene, which is a marker gene for the JA-dependent defense pathway (Fig. 4). In previous work, we demonstrated that *P. aphidis* also can activate the SA-dependent marker gene *PR1* in *Arabidopsis* plants. In contrast to the effect observed in tomato plants, in *Arabidopsis* plants, the JA-dependent marker gene *PDF1.2* was also activated (12). It has been previously shown that beneficial microorganisms can either

activate PR genes directly before pathogen attack (14,71), as we demonstrated with *P. aphidis*, or can prime the plant without gene activation, resulting in rapid and strong PR gene activation upon pathogen attack (17,67,70). The efficacy of *P. aphidis* for control of *C. michiganensis* in tomato plants (cultivar 870) correlated with the synergistic activation of the *PR1a* gene in *P. aphidis*-treated plants that were inoculated with *C. michiganensis* (Fig. 4). These findings are in agreement with the current understanding that biotrophic bacterial resistance involves the activation of genes from

Table 2. Effect of *Pseudozyma aphidis* treatment on severity of disease symptoms caused by *Clavibacter michiganensis*^z

Treatments	DSI (%)	Stem length (cm)
Uninoculated control	0	36.9 ± 3.5 a
Inoculated, <i>P. aphidis</i> treated	50.3*	28.7 ± 7.9 b
Inoculated, control, water treated	76.9	22.9 ± 4.2 c

^z Disease severity index (DSI) was calculated 28 days postinoculation, as described by Raupach et al. (57). Means and standard deviations of stem length followed by different letters are significantly different according to the Tukey-Kramer honestly significant difference test; $P < 0.05$. Means of DSI followed by an asterisk is significantly different from the control water-treated inoculated plants according to Student's *t* test; $P < 0.05$. $n = 10$.

Fig. 3. Effect of *Pseudozyma aphidis* (PA) on the severity of disease induced by *Clavibacter michiganensis* (CM). The frequency of healthy, sick, and dead plants among the groups of plants inoculated with the pathogen with or without the PA treatment ($n = 15$ for each treatment) was calculated 28 days post inoculation. Shown here are the results of one experiment that was representative of at least four experiments with similar results.

Fig. 4. Resistance induced by *Pseudozyma aphidis* (PA). Wild-type tomato plants were sprayed with a suspension of PA spores (10^8 spores/ml). LoxD (LOX) and *PR1a* gene expression was monitored using quantitative real-time polymerase chain reaction. **A**, Relative gene expression at 3 days after the application of PA, relative to that observed in water-treated plants (control). **B**, Relative gene expression at 28 days after *Clavibacter michiganensis* (CM) was applied to plants treated with live *P. aphidis* (PA) or dead autoclaved *P. aphidis* (PA-A) as compared with water-treated plants (control). Relative gene expression (RQ) was calculated using the 2^{-ΔΔCt} method. Results represent means ± standard error ($n = 3$). Shown here are the results of one experiment that was representative of at least four experiments with similar results.

Table 3. Effect of *Pseudozyma aphidis* treatment on severity of disease symptoms induced by *Clavibacter michiganensis* in wild-type (WT) and *NahG* mutant plants^z

Treatment	DSI (%)		Stem length (cm; mean ± SD)	
	WT	<i>NahG</i>	WT	<i>NahG</i>
Inoculated, PA-treated	35.5	35	56.6 ± 2.5	57.6 ± 5.8
Inoculated, control water-treated	47*	51	45.2 ± 4.6	48.1 ± 6.2

^z Disease severity index (DSI) was calculated 42 days postinoculation, as described by Raupach et al. (57). Means and standard deviations (SD) of stem length followed by different letters are significantly different according to the Tukey-Kramer honestly significant difference test, $P < 0.05$. Means of DSI followed by asterisk is significantly different from control according to Student's *t* test, $P < 0.05$. $n = 10$.

the SA-dependent pathway (45). However, we cannot rule out the possibility of the involvement of other, as-yet-unidentified SA-independent PR genes and other hormonal pathways activation after *P. aphidis* application. Indeed, when we treated *NahG*-transgenic tomato plants (Money Maker background), which do not accumulate SA and do not activate PR1a, with *P. aphidis*, we observed a reduction in the severity of disease induced by *C. michiganensis* (Table 3). This indicates that the inhibition of *C. michiganensis* by *P. aphidis* is SA-independent in Money Maker background and probably involves genes other than PR1a, such as GlucB, PTI5, and PR5 that were upregulated in this cultivar. Furthermore, PR1a upregulation could not be demonstrated after *P. aphidis* application in *NahG* plants, as expected, but it was also lower in their corresponding wild-type background Money Maker as compared with cultivar 870. This is consistent with a previous observation that demonstrated that different tomato cultivars respond differently to the biocontrol agent *Trichoderma* and activate different sets of PR genes (65). It is also a possibility that other PR genes and hormonal pathways, as-yet-unidentified, are also involved in the defense response against *C. michiganensis* after *P. aphidis* application in both cultivars. Using *NahG*-transgenic *Arabidopsis* plants infected with *B. cinerea*, we previously suggested that *P. aphidis* either bypasses the SA pathway to activate downstream PR genes or activates other as-yet-undiscovered defense pathways (12). Alternatively, the systemic resistance in an SA-independent manner can also be activated without activation of PR gene expression as also suggested by other works using the biocontrol agent *Pseudomonas fluorescens* (8,52–54,58,61) and for rhizobacterial species (8,69,73). Involvement of an SA-independent pathway in induced-resistance activation has been shown before using the biocontrol agent *T. harzianum* (41,50). On the other hand, some beneficial microorganisms activate SA-dependent induced resistance (1,19), but different pathways might also be involved (36,37,68). Recent work demonstrated that *C. michiganensis* activated the ET defense pathway in tomato plants (7). We also demonstrated activation of the ET-dependent PTI5 after *P. aphidis* treatment in both cultivars but it was not upregulated in cultivar 870 after infection with *C. michiganensis*. Furthermore, PTI5 was not upregulated also in *NahG* plants as was expected according to the previous results, suggesting that SA suppresses PTI5 activation (31). Differences in plant genotypes used in the various studies might be responsible for the alterations in defense responses. As *C. michiganensis* is mainly a systemic pathogen, it is likely that much of the biocontrol effect we observed using the epiphytic *P. aphidis* resulted from activation of induced resistance. Nevertheless, we cannot rule out the possibility of a direct antibiosis effect achieved during pathogen inoculation and dispersal via wounds and natural openings. In summary, in this article, we demonstrate the ability of *P. aphidis* to reduce the incidence of infection of tomato plants by *C. michiganensis* and to activate the plant's defense system in an SA-independent manner.

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