Phyllosphere Colonization by a Soil *Streptomyces* sp. Promotes Plant Defense Responses Against Fungal Infection

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Streptomyces are soil-dwelling, filamentous actinobacteria and represent a prominent bacterial clade inside the plant root microbiota. The ability of streptomycetes to produce a broad spectrum of antifungal metabolites suggests that these bacteria could be used to manage plant diseases. Here, we describe the identification of a soil *Streptomyces* strain named AgN23 which strongly activates a large array of defense responses when applied on *Arabidopsis thaliana* leaves. AgN23 increased the biosynthesis of salicylic acid, leading to the development of *salicylic acid induction deficient 2 (SID2)*-dependent necrotic lesions. Size exclusion fractionation of plant elicitors secreted by AgN23 showed that these signals are tethered into high molecular weight complexes. AgN23 mycelium was able to colonize the leaf surface, leading to plant resistance against *Alternaria brassicicola* infection in wild-type *Arabidopsis*

plants. AgN23-induced resistance was found partially compromised in salicylate, jasmonate, and ethylene mutants. Our data show that *Streptomyces* soil bacteria can develop at the surface of plant leaves to induce defense responses and protection against foliar fungal pathogens, extending their potential use to manage plant diseases.

Keywords: *Alternaria brassicicola*, *Arabidopsis thaliana*, bacteria–plant symbiosis, biocontrol, defense signaling pathways, ethylene, jasmonate, plant defense, salicylic acid, *Streptomyces*

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In their environment, plants have to cope with a wealth of beneficial, commensal, and pathogenic microorganisms (Berendsen et al. 2012; Vorholt 2012). Microbial consortia living around plants can alter disease progression, and manipulating the plant microbiota holds great promise to develop biocontrol solutions (Busby et al. 2017). Interactions between plants, pathogens, and the microbiota range from indirect exchange of extracellular bioactive compounds to spatial and nutrient competition around and inside plant tissues (Chaparro et al. 2013, 2014; Stringlis et al. 2018b). Unlike pathogens, beneficial microbes can live inside plant tissues (endophytes) or at the surface (rhizoplane) or around (rhizosphere) plants roots and leaves without causing symptoms (Bergelson et al. 2019). The activation of plant defense by such microbes often enhances disease resistance to subsequent pathogen invasion (Bhattacharyya and Jha 2012). Efficient plant defense mechanisms include preexisting physical and chemical barriers as well as immune responses triggered by the perception of molecular patterns exposed by pathogens upon plant colonization (van Loon et al. 1998). Pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns such as flagellin, elongation factor, or peptidoglycans interact with dedicated plant cell receptors, the pattern recognition receptors, which activate PAMP-triggered immunity (PTI) responses (Boller and Felix 2009; Stringlis et al. 2018a; Zipfel 2008). The activation of hallmark PTI marker genes is largely dependent on salicylic acid (SA) signaling; nonetheless, defense responses also involve interplays with jasmonic acid (JA) and ethylene (ET) signaling and biosynthesis (Pieterse et al. 2012). In *Arabidopsis*, SA biosynthesis by the isochorismate synthase gene *salicylic acid induction deficient 2 (SID2)* mediates most of the SA biosynthesis in the plant in stress responses (Ferrari et al. 2003;

Wildermuth et al. 2001). SA-dependent responses are prominently active against biotrophic pathogens and are associated with the local and systemic induction of pathogenesis-related (PR) proteins such as PR1 (Bürger and Chory 2019). By contrast, the defense pathways regulated by JA or ET are particularly important for the resistance against necrotrophic pathogens and interact antagonistically with the SA pathway (Bürger and Chory 2019). Collectively, the balance between these pathways enables plant resistance to most foliar pathogens. It is noteworthy that these disease resistance mechanisms can be activated locally as well as systemically by JA for induced systemic resistance and SA for systemic acquired resistance (Carvalhais et al. 2015; Pieterse et al. 2014; van Loon et al. 1998; Wang et al. 2018).

Modern agriculture faces the challenge to reduce pesticide use while pests develop resistance mechanisms to chemical active ingredients. New discoveries of fungicidal substances with acceptable ecotoxicological and toxicological features are becoming rare. Therefore, the use of microbial strains or the design of synthetic bacterial communities as biocontrol agents could represent an attractive alternative to ensure high yield (Syed Ab Rahman et al. 2018). Although a number of bacterial and fungal strains are currently included in commercial preparations aiming to protect plants against diseases (Bhattacharyya and Jha 2012; Syed Ab Rahman et al. 2018), identification of new beneficial microbial species is required to optimize this approach.

Among the most promising candidates are Gram-positive bacteria belonging to the genus *Streptomyces*, which constitutes a prominent component in the plant rhizosphere (Lundberg et al. 2012; Rey and Dumas 2017; Viaene et al. 2016; Vurukonda et al. 2018). These filamentous bacteria inhabit both soils and aquatic environments and can disseminate in the air (Palaniyandi et al. 2013; Rey and Dumas 2017; Sarmiento-Vizcaíno et al. 2016; Schrey and Tarkka 2008). Streptomycetes are extremely rarely pathogens (Armijos-Jaramillo et al. 2017; Joshi et al. 2007); by contrast, they are beneficial or commensal symbionts in a wide range of plants and invertebrates (Seipke et al. 2012). Numerous examples of plant-colonizing *Streptomyces* spp. have been reported (Cao et al. 2016; Hassan et al. 2017; Li et al. 2018; Mingma et al. 2015; Ondrejčková et al. 2016; van der Meij et al. 2018). Rhizospheric and endophytic *Streptomyces* spp. are often able to modulate plant growth (Niu et al. 2016; Olanrewaju and Babalola 2019; Vurukonda et al. 2018), potentially by producing diverse phytohormones (Rashad et al. 2015). Complementarily, streptomycetes isolated in natura from *Arabidopsis* respond to plant hormones by producing specialized secondary metabolites (van der Meij et al. 2018). *Streptomyces* spp. also interact in the rhizosphere with other microbes, thereby suppressing plant diseases caused by telluric pathogenic fungi (Bakker et al. 2014; Cordovez et al. 2015; Kinkel et al. 2012; Schrey and Tarkka 2008). As an example, the hallmark antibiotic streptomycin is strongly produced by a rhizospheric *Streptomyces* strain during *Arabidopsis* bolting and flowering, presumably protecting plants from pathogens (Chaparro et al. 2014). By contrast, other *Streptomyces* strains support growth of ectomycorrhizal fungi (Riedlinger et al. 2006; Schrey et al. 2005, 2007; Tarkka et al. 2015). Although *Streptomyces* spp. are prominently found in roots, a few examples of leaf colonizers have been reported; however, this type of interaction has been widely overlooked (Mingma et al. 2015; Shimizu et al. 2000).

Hitherto, most *Streptomyces* spp. evaluated as biocontrol agents were solely screened based on their antifungal properties, neglecting the potential of plant defense stimulation raised by root colonization (Conn et al. 2008; Hamed and Mohammadipanah 2015; Shao et al. 2018). In agriculture,

attempts to use streptomycetes for biocontrol of fungal diseases led to the development of soil amendment products such as Actinovate (*Streptomyces lydicus* WYEC 108) and Mycostop (*S. griseoviridis* K61). However, *Streptomyces* spp. application into soils remains a technical bottleneck and applying *Streptomyces* spp. as foliar treatment is a desirable approach. In this work, we report on the biological activities of a new *Streptomyces* strain on *Arabidopsis*. We show that this strain is able to transiently develop at the surface of plant aerial tissues and produce extracellular elicitors of plant defense reactions, leading to protection against fungal pathogens.

RESULTS

Streptomyces AgN23 produces elicitors leading to induction of PR1 gene expression in *Arabidopsis* seedlings among a range of *Streptomyces* spp.

In a search of new soil bacteria stimulating plant defense responses, soil samples were collected from the rhizosphere of field-located grape roots and 32 actinomycete strains were isolated. The screening of these strains was based on the induction of *PR1* gene expression, using a reporter line expressing the β -glucuronidase gene *uidA* (hereafter referred to as *GUS*) under the control of the *PR1* promoter (Shapiro and Zhang 2001). The 32 actinomycetes were grown in liquid flasks for 1 week before the culture media was collected and applied to hydroponically grown *Arabidopsis* Col-0 *PR1::GUS* at a final concentration of 1% (Fig. 1A). Based on these results, the strain AgN23 was selected, considering its specific ability to strongly activate the *GUS* gene. Analysis of the AgN23 16S ribosomal RNA (rRNA) sequence (GenBank JN104730.1) revealed a strong homology with *S. castelarensis*, belonging to the *S. violaceusniger* 16 rRNA clade (Supplementary Dataset S1) (Goodfellow et al. 2007; Kumar and Goodfellow 2008; Kumar et al. 2007). Then, induction of the *GUS* gene was validated on plants grown in pots and treated with AgN23 extracellular culture medium harvested after 1 to 6 days of culture. To address the duration of defense induction following initial treatment of plants grown in pots, we sprayed leaves with mock media (0 day) and AgN23 culture media harvested after 1, 2, 4, and 6 days of culture, then tracked the *GUS* activity in *Arabidopsis* 5, 7, or 14 days posttreatment (hpt) (Fig. 1B). The elicitor activity released by the bacteria reached its maximum after 6 days of culture, resulting in transient induction of plant defense, which gradually decreased after 5 days (Fig. 1B).

Streptomyces AgN23 produces extracellular compounds inducing a broad spectrum of immune responses in *Arabidopsis* seedlings.

An RNA sequencing (RNAseq) approach was devised to decipher earlier events in plant response to AgN23 extracellular culture media and get a comprehensive view of plant immunity activation. *Arabidopsis* seedlings were immersed in Murashige and Skoog (MS) medium containing the AgN23 culture media and harvested 1 or 6 hpt. Pairwise comparisons between biological triplicates of treated and untreated plants were calculated at each time point of the kinetic to identify differentially expressed genes (Supplementary Dataset S2). Genes showing a fold change (FC) greater than 1 or less than -1 in a $\log_2$ base and a false discovery rate (FDR) < 0.001 were considered for subsequent analyses (Fig. 2A; Supplementary Datasets S2 and 3). This dataset was validated by selecting a set of 28 genes transcriptionally regulated in the RNAseq data and confirming their induction by reverse-transcription quantitative PCR (RT-qPCR) ($R^2 = 0.77$) (Fig. 2B). At 1 hpt, we found 249 regulated genes, among which 74% were induced (Supplementary Dataset S3). Similarly, of 1,304 regulated genes at 6 hpt, 987

genes showed induction (Supplementary Dataset S4). The examination of commonly induced genes at 1 or 6 hpt showed that their induction was stronger at 6 hpt (130 of 139) (Fig. 2C; Supplementary Dataset S5). These data suggest that the *Streptomyces* AgN23 supernatant triggered a quick induction of plant responses, gradually increasing over time.

To get a first hint into the processes affected by bacterial supernatant treatment, we characterized the 485 genes showing $\log_2$ FC > 2 at 6 hpt using the gene ontology (GO) classification based on the *Arabidopsis* TAIR10 genome. These genes were classified in 117 different GO terms belonging to “Biological Processes”. GO term enrichment analysis was then summarized and visualized using REVIGO (Fig. 2D; Supplementary Dataset S6) (Supek et al. 2011). The analysis emphasized a cluster around the “defense response” GO term. Other close and highly significantly represented GO terms are “immune response”, “response to stress”, “response to external stimulus”, “response to organonitrogen compound” that is the parent GO term of “chitin response”, and “hormone response”. Similarly, we detected strong enrichment in “response to external biotic stimulus” that is the parent GO terms of “response to bacterium”, “response to fungus”, and “systemic acquired resistance”. Other enriched GO terms were linked to disease resistance; notably, GO terms corresponding to lignin and phenylpropanoid pathways and secondary metabolism components such as “phytoalexin biosynthetic” that are collectively child terms of the parent GO “toxin metabolic process” (Fig. 2D).

Among the 241 genes showing the strongest $\log_2$ FC > 3 at 6 hpt, 83 genes had a predicted function, most of them being related to defense responses (Supplementary Dataset S7). This analysis revealed the induction of a large number of putative receptor genes, transcription factors, and genes involved in signaling pathways, oxidative stress, secondary metabolism and cell walls, and PR proteins. Interestingly, treatments with AgN23 extracellular medium strongly induced several genes encoding P450 enzymes involved in glucosinolate metabolism, notably leading to the synthesis of camalexin, a major antimicrobial compound (Rajniak et al. 2015). Biosynthetic genes

were found to be coordinately expressed with the gene coding a MYB transcription factor (MYB122) involved in the regulation of camalexin biosynthetic genes (Frerigmann et al. 2015). Overall, transcriptomic data strongly support the intense activation of plant defense responses mounted by AgN23-derived compounds released in the culture supernatant.

***Arabidopsis* foliar responses to perception of AgN23-derived compounds and bacterial cell inoculation.**

To address whether adult Col-0 rosettes grown in pots also respond to AgN23, we analyzed the immune responses induced by extracellular compounds or viable bacterial cells following foliar treatments. Expression of immune responses was monitored with 58 markers corresponding to housekeeping genes and various classes of immune and hormonal responses previously validated to investigate *Arabidopsis* defense responses (Vergnes et al. 2014). Treatments with 10-fold dilution of culture media were able to activate diverse defense processes, with the exception of JA-responsive genes (Fig. 3). By contrast, JA genes (*ATHCHIB*, AT3G12500; and the defensin *PDF1.2*, AT5G44420) were strongly induced by bacterial cell treatment (Fig. 3). Also, we observed that JA-related responses triggered by bacterial cells did not compromise the activation of SA-responsive genes (*wall-associated kinase 1*, AT1G21250; *PR1*, AT2G14610; *PR5*, AT1G75040; *WRKY70*, AT3G56400; and *ANKYRIN*, AT5G54610) (Fig. 3). Taken together, these data confirmed that AgN23 can modulate immune responses in adult plants and suggests that bacterial cell treatment leads to co-incident SA- and JA-related plant responses.

***Streptomyces* sp. AgN23 produces extracellular necrotic elicitors present in high molecular weight fractions.**

To characterize the compounds released by AgN23 in its extracellular culture medium, we performed a sequential molecular weight size fractionation of a 5-day-old culture by membrane filtration. Six fractions of increasing molecular weight (<5 kDa to >100 kDa) were sprayed on *PR1::GUS* plants grown in pots. We observed that only a high molecular weight fraction (>100 kDa) induced *GUS* expression (Fig. 4A).

Fig. 1. Production of plant defense elicitor by *Streptomyces* AgN23 strain. **A**, Screening of bacterial culture media of 32 *Streptomyces* sp. strains cultivated for 6 days by treatment in hydroponic condition of the *Arabidopsis thaliana* Col-0 *PR1::GUS* line and measurement of β -glucuronidase (GUS) activity 2 days posttreatment (dpt). **B**, Samples of the culture media were harvested at 1, 2, 4, and 6 days of cultivation and used for foliar treatment of Col-0 *PR1::GUS* *Arabidopsis* seedlings grown for 3 weeks prior to treatment. GUS enzymatic activity was recorded at 5, 7, and 14 dpt. One-way and two-way analysis of variance followed by Dunnett's multiple comparison test were performed for A and B, respectively, to identify results statistically divergent from the control conditions (*, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively). Error bars represent the standard error to the mean and $n = 3$ for each condition.

During these experiments, leaves treated with AgN23 culture medium frequently developed necrotic spots 48 hpt (Fig. 4B) which could be stained with Trypan Blue (Fig. 4C), suggesting that necrotic elicitors are produced by AgN23. To determine whether the development of necrotic lesions was due to the production of phytotoxic compounds or the consequence of a strong induction of defense responses, *sid2-2* plants, which are unable to form an SA-dependent cell death response, were treated with AgN23 culture filtrate or cells. No lesion was observed on *sid2-2* leaves (Fig. 5A), suggesting that accumulation of SA is required for their formation. Determination of total SA concentration in treated leaves confirmed that treatment with AgN23 culture medium or AgN23 cells strongly induced the synthesis of SA, which can act as a signal for the development of a programmed cell death leading to the formation of hypersensitive-like lesions (Fig. 5B).

Leaves surface colonization by AgN23 induces resistance to fungal pathogens.

The behavior of AgN23 on leaf surfaces was followed by scanning electron microscopy at various times upon spraying of AgN23 bacterial suspension. Bacterial hyphae rapidly colonized the surface, including trichomes (Fig. 6A). In some cases, filaments terminated with vesicle-like structures referred to as sporangium in some actinobacteria (Fig. 6B) (Barka et al. 2015). Similarly to previous phyllosphere colonization reports of *Streptomyces* spp., AgN23 colonized stomata and penetration attempts induced local necrotic responses (Fig. 6C) (Minamiyama et al. 2003). To evaluate the protective effect of AgN23 on plant disease, *Arabidopsis* mutant lines defective in immunity hormone signaling were compared. In addition to *sid2-2*, mutant lines tested included *jasmonate resistant 1 (jar1-1)*, which is deficient for the jasmonate-amido synthetase

Fig. 2. Transcriptional responses to treatment with AgN23 culture media. **A**, Number of genes showing transcriptional induction (+) or repression (–) based on log₂ fold change (FC) > |1| and a false discovery rate-corrected *P* value < 0.001. Red bars = upregulated genes and green bars = downregulated genes. **B**, Validation of RNA sequencing (RNAseq) results by reverse-transcription quantitative PCR (RT-qPCR). **C**, Comparison of pool of genes regulated by AgN23 culture media treatment at 1 and 6 h posttreatment (hpt). **D**, REVIGO semantic plot of gene ontologies corresponding to the gene set showing log₂ FC > |1| at 6 hpt. Each condition corresponds to *n* = 3 biological replicates.

converting JA to isoleucine-jasmonate, and *ethylene insensitive 2* (*ein2*), which is deficient for ET perception (Alonso et al. 1999; Ferrari et al. 2003; Kawamura et al. 2009; Li et al. 2015; Varma Penmetsa et al. 2008; Wang et al. 2007). Aerial tissues of the wild-type and mutant lines were sprayed with bacterial biomass and inoculated 48 h later in a detached-leaf assay with the necrotrophic pathogen *Alternaria brassicicola*. AgN23 treatment resulted in a strong decrease of visual necrotic lesions at 6 days postinoculation (dpi) in both the wild type and all mutants (Fig. 7). In support of this observation, we carried out Trypan Blue staining at the end of the assay and observed a strong blue coloration caused by *A. brassicicola* necrotrophic colonization in the leaf center of control plants. By contrast, Col-0 and mutant plants treated with AgN23 showed discrete necrotic regions similar to those observed in Figure 4C, and this pattern was conserved upon subsequent inoculation with the fungus, suggesting that AgN23 prevented the establishment of necrotrophic colonization. Complementarily, the fungal development was quantified by counting conidia produced on the leaves at 6 dpi. In the Col-0 line, reduced amounts of conidia were observed upon treatment with AgN23. However, this sporulation was not significantly affected in *sid2-2*, *jar1-1*, and *ein2* mutants (Fig. 8), suggesting that induction of defense responses involving SA and JA or ET pathways could participate in the restriction of fungal development. Taken together, these results suggest that, although protection in AgN23-treated leaves mostly relies on plant defense responses, we cannot rule out direct effects of the *Streptomyces* strains on *A. brassicicola* development on the phylloplane.

DISCUSSION

By screening a selection of 32 *Streptomyces* strains isolated from soil, we identified one strain, termed AgN23, which we found to be a strong and selective inducer of the *PR1* gene in *Arabidopsis thaliana* among all the strains tested. To decipher plant responses to AgN23, we used transcriptomic study of *A. thaliana* responses to AgN23 culture media. We showed that AgN23 culture media can trigger a broad range of plant immune responses, including JA- and SA-dependent responses. Hydroponic treatment of whole seedlings with the bacterial extracellular medium led to the regulation of more than 1,000 plant genes. GO analysis showed that a large number of induced genes fall into the immunity-related functional classes and, coincidentally, raised SA- and JA-related responses (Betsuyaku et al. 2018). Interestingly, AgN23 induced accumulation of SA and SA-dependent necrotic lesions on plant leaves, and microscopic examination of leaf surfaces colonized by AgN23 mycelium revealed the presence of localized stomatal cell death at the site of attempt of penetration. Taken together, our results suggest that AgN23 produces necrotic elicitors, leading to SA-dependent necrotic response (Lee et al. 2017).

Among the 32 actinomycetes isolates tested, the specificity of PR1 induction following treatment with AgN23 culture media suggests that the strain produces specific compounds either absent or not expressed in the other strains during microbiological culture. Although the biochemical nature of elicitors produced by AgN23 remains to be identified, streptomycetes are renowned to secrete extracellular enzymes as well as a large array of antifungal and antibacterial small specialized metabolites (Chater et al. 2010). However, only a few of them have been studied for their ability to induce plant immunity. This is the case for the cellulose synthase inhibitor thaxtomin A produced by a phytopathogenic strain, *S. scabies*. Thaxtomin A has been shown to induce phytoalexin synthesis in *Arabidopsis* and tobacco and other immune responses such

as cell death or calcium fluxes (Duval and Beaudoin 2009; Joglekar et al. 2018; Lerat et al. 2009). These compounds are genetically encoded into specialized metabolite biosynthetic gene clusters such as nonribosomal peptide synthase (NRPS) and polyketide synthase (PKS) (Chandra and Chater 2014;

Fig. 3. Heatmap display of *Arabidopsis* genes regulation following treatment with AgN23 culture media (CM) or bacterial cells (Bact.). Plants grown in pots were treated by foliar spray and a set of 58 genes was studied by reverse-transcription quantitative PCR. The selection comprised housekeeping genes (HK); genes induced by phytohormones (salicylic acid [SA] and jasmonic acid [JA]), microbial pathogen-associated molecular patterns (flagellin [flg22], chitosaccharides [COS], harpin, and lipopolysaccharides [LPS]); genes expressed upon pathogen infection (biotrophic and necrotrophic); and nonhost recognition. Genes are identified according to the TAIR10 *Arabidopsis* genome nomenclature. Differentially expressed genes are displayed as log₂ fold change of treated versus untreated samples at each time point (1, 6, and 24 h). Genes showing absence of regulation are in black and genes showing unreproducible expression values are displayed in white on the heatmap. Each datapoint corresponds to *n* = 3 biological replicates.

Liu et al. 2013; Niu et al. 2016). Streptomycetes can harbor contrasting lifestyles between axenic microbiological and environmental culture conditions (Jones et al. 2017). This suggests that the differences observed in defense responses following leaf treatment with culture media (inducing SA responses) and bacterial cells (inducing SA and JA responses) may be linked to differences in AgN23 elicitors released in culture media. According to our size fractionation approach, it is conceivable that elicitors are large molecular weight components of AgN23 such as peptidoglycans or other components of Gram + cell walls. However, such signals may also have been released by the other strains tested in our initial screening. Alternatively, specialized metabolites produced by NRPS or PKS are usually in the range of 0.1 to 3 kDa but may be enclosed in large vesicular structures secreted in the media (Chater et al. 2010; Hoefler et al. 2017; Schrempf and Merling 2015; Schrempf et al. 2011).

To evaluate the effect of the induction of immune responses on the interaction with a fungal pathogen, AgN23-treated leaves were inoculated with the necrotrophic pathogen *Alternaria brassicicola*. Treatment resulted in decreased onset of visual symptoms in both Col-0 and immunocompromised mutants *sid2*, *ein2*, and *jar1*. However, the level of spores produced by the fungus 6 dpi was significantly reduced in Col-0 plants but not in *sid2*, *jar1*, and *ein2* plants. This finding

suggests that plant defense activation by AgN23 is not involved in the development of disease symptoms but may prevent the completion of fungal multiplication. This also suggests that other mechanisms such as the production of antifungal metabolites participate in the AgN23-induced protection. Because *Streptomyces* spp. are primarily present around plant root tissues, most studies focused on systemically induced resistance in diverse annual crops (Cao et al. 2016; Lehr et al. 2008; Zhao et al. 2012) or trees (Kurth et al. 2014). Nonetheless, a few reports showed that streptomycetes can also dwell in plant leaves (Mingma et al. 2015; Shimizu et al. 2000). Our study illustrates how a strain isolated from soil can develop on plants' aerial parts and locally activate defense responses, extending the potential use of *Streptomyces* spp. to protect plants against foliar diseases.

Engineering the plant microbiota is a cornerstone for the development of agronomic strategies to protect plants from diseases without chemical pesticides. Streptomycetes are major bacterial colonizers of plant roots and important microbial components of disease-suppressive soils. These bacteria are prolific producers of specialized metabolites with antibacterial and antifungal features. However, streptomycetes rarely colonize plant leaves; thus, their potential to protect crops from aerial pathogens has been neglected. We showed how a *Streptomyces* strain isolated from plant roots can be used to trigger

Fig. 4. AgN23 releases elicitors associated with high molecular weight fractions. Five-day-old culture media of AgN23 were subjected to sequential ultrafiltration ranging from >100 kDa to >5 kDa. Fractions were sprayed on the *PRI::GUS* reporter line grown in pots. **A**, β -Glucuronidase (GUS) enzymatic activity was measured in plants 48 h after being sprayed with the fractions. Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparison test (**** indicates $P < 0.0001$). Bars represent the standard error to the mean; each test was conducted with $n = 3$ biological replicates. **B**, Mock and >100 kDa fraction-treated Col-0 *PRI::GUS* plants observed 48 h after treatment. AgN23 high molecular weight fractions induced discrete dry lesions. **C**, Trypan Blue staining of leaves treated with the fractions. Note the necrotic lesions following treatment with the high molecular weight fraction. Scale bars = 1 cm.

potent local defense responses in plant leaves and restrict colonization by fungal pathogens. This finding illustrates how soil microorganisms may be introduced into the plant phyllosphere microbiota to achieve crop protection.

MATERIALS AND METHODS

Arabidopsis thaliana lines and cultivation methods.

Arabidopsis thaliana lines used were Col-0 wild-type, transgenic line *PR1::GUS* (Shapiro and Zhang 2001), *sid2-2* (Wildermuth et al. 2001), *jar1-1* (Staswick et al. 1998), and *ein2* (Alonso et al. 1999). For in vitro assays, seed were soaked in water for 40 min, then treated for 30 min in 2.4% sodium hypochlorite solution, followed by four washings in sterile water. The surface-sterilized seed were transferred into wells of 24-well microtiter plates (2 seeds/well), containing 300 µl of MS medium (Sigma-Aldrich, Saint-Quentin-Fallavier, France) supplemented with 1% sucrose. The plates were incubated under a cycle of 16 h of illumination and 8 h of night at 23°C on a rotary shaker at 90 rpm for 3 days, then on a static tray for 12 days. For SA analysis and pathogenicity tests, plants were grown on soil (PROVEEN; Bas Van Buuren B.V., Holland) in a growth chamber under 12 h of light at 23°C and 12 h of darkness at 20°C for 3 weeks. Foliar treatments of 3-week-old plants grown in pots were performed by spraying the compounds at 1 ml/pot using a manual spray. For all the treatments, a wetting agent (Actilandes; ActionPin Cazalieu, Castets, France) was added at a final concentration of 1 µl/liter.

Alternaria brassicicola inoculation assay.

Alternaria brassicicola strain Abra43 (Sellam et al. 2007) was maintained on potato dextrose agar. For pathogenicity tests, fungal spores were harvested and adjusted to 5×10^5 spores/ml in a Fuchs-Rosenthal counting cell (Pochon et al. 2012). Detached leaves of *Arabidopsis thaliana* were deposited on the agar medium and inoculation of the fungus was done by depositing a drop of 10 µl on each leaf. The plates were then parafilmed to maintain high hygrometry and kept in a climate chamber for 6 days, according to the cycle 16 h in the light at 22°C and 8 h in the dark at 20°C. At 6 dpi, the spores formed on symptomatic leaves were harvested by shaking three leaves in 15-ml Falcon tubes containing 3 ml of 0.1% Tween 20 solution. The leaves were removed from the tubes and detached spores were pelleted by centrifugation (15 min at $5,000 \times g$). The supernatant was discarded and spores resuspended in 12 µl of 0.1% Tween 20 solution before counting in the Fuchs-Rosenthal cell.

AgN23 cultivation methods

and high molecular elicitor fractionation.

The AgN23 strain was maintained and cultivated as described by Errakhi et al. (2007) and patents WO2015044584 and WO2015044585. In brief, the supernatant of AgN23 was produced from liquid shaken culture of AgN23 grown for 6 days in 250-ml flasks containing 50 ml of Bennet medium. The bacterial biomass was separated from supernatant by centrifugation and the supernatant directly used for hydroponic plant treatment. For size exclusion fractionation of the elicitor activity, the supernatant was sequentially centrifuged on Vivaspin (Sartorius, Aubagne, France) ultrafiltration columns with molecular weight cut-offs of 5 to 10, 10 to 50, and 50 to 100 kDa, according to manufacturer recommendations. The eluent of 5-kDa columns and the retentate of the 50- to 100-kDa cut-off were also considered for assessment of biological activities. For inoculation of bacterial biomass on *Arabidopsis* plants, 1 ml of a 7-day-old culture broth in liquid Bennet medium was directly sprayed on plants.

GUS activity assays.

The assays were conducted as described by Vergnes et al. (2014). Briefly, total proteins were extracted from 100 mg of treated plants after grinding in liquid nitrogen, with 100 µl of

Fig. 5. AgN23 culture medium induces necrotic lesions triggered by the accumulation of salicylic acid (SA). **A**, Trypan Blue staining of leaves from wild-type Col-0 and the *sid2-2* line; no lesions were observed on the *sid2-2*-treated leaf (scale bar = 1 cm). **B**, Quantification of total SA in Col-0 and *sid2* at 1 day posttreatment (dpt) following treatment with *Streptomyces* sp. AgN23 cell suspension; $n = 2$ independent biological replicates displayed separately by black and white dots.

GUS buffer (100 mM phosphate buffer [pH 7], 0.1% TritonX-100, and 10 mM β -mercaptoethanol). To quantify proteins, 200 μ l of Bradford reagent (Bio-Rad Laboratories, Marnes-la-Coquette, France) was added to 10 μ l of samples. After incubation (15 min at 25°C), absorbance was measured at 565 nm. Standard curve was done with 1 to 20 μ g of bovine serum albumin (Sigma-Aldrich). Glucuronidase activity was determined with 25 μ l of protein extracts and 1 mM 4-methylumbelliferyl glucuronide (MUG) (Sigma-Aldrich) in a total reaction volume of 200 μ l. Fluorescence generated by MUG metabolism was measured every 5 min during 120 min on a TriStar LB 941 Multimode Microplate Reader (Berthold Technologies, Thoiry, France) at 37°C with excitation of 360 nm and emission of 460 nm. Glucuronidase activity was calculated from the linear part of the reaction (between 20 and 100 min) and normalized as nanokatal per milligram of total proteins.

Genomic DNA extraction and PCR amplification for AgN23 16S ribosomal DNA sequencing.

Genomic DNA was extracted from AgN23 mycelium after centrifugation of a 48-h liquid culture. Extraction was done using NucleoBond AXG columns and NucleoBond Buffer Set III (Macherey Nagel, Hoerd, France). The 16S ribosomal DNA was amplified from gDNA by PCR using 5'-AGAGTTTG ATCCTGGCTCAG-3' and 5'-GGTTACCTGTTACGACTT-3' primers. Sanger sequencing was performed at the GeT-PlaGe core facility, Toulouse, France.

RNA isolation and analysis of gene expression by RT-qPCR.

Total RNA was extracted using the SV Total RNA Isolation System kit (Promega, Charbonnières, France). For each sample, 1 μ g of total RNA was reverse-transcribed with the High-Capacity cDNA Reverse-Transcription Kit (Applied Biosystems, Courtaboeuf, France). High-throughput qPCR was performed using the BioMark HD System (Fluidigm, Issy les Moulineaux, France). Briefly, cDNAs were diluted to approximately 50 ng/ μ l prior to being submitted to specific target amplification by 14 cycles of PCR amplification (95°C for 15 s and 60°C for 4 min) in a reaction mix containing the 96 primer pairs (50 nM) and the TaqMan PreAmp Master Mix (1:2) (Applied Biosystems). Primer sequences are available in supplementary materials from Vergnes et al. (2014). Preamplified cDNAs were diluted with Tris-EDTA buffer (1:5) and used for

qPCR array analysis in a reaction mix containing TaqMan Gene Expression Master Mix, DNA Binding Dye Sample Loading Reagent, and EvaGreen. Data were analyzed with the BioMark Real-Time PCR Analysis Software (version 2.0; Fluidigm, Les Ulis, France), The NormFinder software (Andersen et al. 2004) was used to determine the best housekeeping genes and the clathrin gene *AT5G46630* was retained for normalization. Relative gene expression was calculated over three independent experiments and significant gene deregulations were determined by Student's *t* test.

RNAseq experiment and analysis.

The same RNA samples prepared for RT-qPCR were used for RNAseq experiments. RNAseq libraries were prepared according to Illumina's protocols using the Illumina TruSeq Stranded mRNA Sample Prep Kit to analyze mRNA, at the GeT-PlaGe core facility, Toulouse, France (Gaulin et al. 2018). Briefly, mRNA was selected using poly-T beads, cDNA was generated using random hexamer priming, and adapters were then added. Ten cycles of PCR were applied to amplify the libraries. Library quality was assessed using an Agilent Bioanalyzer, and the libraries were quantified by qPCR using the Kapa Library Quantification Kit. RNAseq experiments were performed on an Illumina HiSeq2500 using a paired-end read length of 2 $\times$ 100 pb with the Illumina TruSeq SBS Sequencing Kits v3. The resulting FASTQ files contained 452,736,864 reads for AgN23 treatment and 415,999,444 reads for the control. The quality control was performed on raw sequencing datasets using FastQC. The paired-end reads were mapped to the *Arabidopsis* TAIR10 genome reference using CLC Genomics Workbench version 8.5.1 with default parameters and expressed as reads per kilobase per million mapped reads (Supplementary Dataset S2). Gene regulation was determined using two-by-two comparisons between Actilandes-treated and AgN23-treated samples at each timepoint with the "Empirical Analysis of Differential Gene Expression" pipeline of the software (Supplementary Dataset S2). Briefly, the test is based on the assumption that the count data follows a negative binomial distribution. The "Exact Test" of Robinson and Smyth is similar to Fisher's exact test but also accounts for overdispersion caused by biological variability. Whereas Fisher's exact test compares the counts in one sample against those of another, the Exact Test compares the counts in one set of count samples against those in another set of count samples. The Exact Test in the EdgeR

Fig. 6. Development of AgN23 on the surface of *Arabidopsis* leaves. **A**, Hyphal development at 7 days postinoculation with a 7-day-old liquid culture on the adaxial face of the leaf. **B**, Growth of the mycelium on a trichome branch; hyphae are terminated by sporangium-like structures (arrowheads). **C**, Cell death response of stomata observed upon colonization by AgN23 hyphae (arrowhead). Scale bars = 25 μ m.

Bioconductor package provides the user with the option to set a large number of parameters. The implementation of the “Empirical analysis of DGE” algorithm in the Genomics Workbench uses, for the most part, the default settings in the edgeR package, version 3.4.0.

In the end, we only considered genes with a twofold induction or repression between treated and untreated situations and a P value < 0.001 after correction with the FDR method developed by Benjamini and Hochberg (Supplementary Dataset

S2). Raw data can be found on the NCBI Gene Expression Omnibus (GSE119986).

Microscopy.

The observation of AgN23 development on *Arabidopsis* leaves was performed using scanning electronic microscopy on an FEG FEI Quanta 250. Leaf samples were placed on the micrometric platen of the scanning electron microscope before being frozen in liquid nitrogen and metallized with platinum.

Fig. 7. AgN23-mediated protection of *Arabidopsis* leaves against *Alternaria brassicicola*. Symptoms were observed at 6 days postinoculation of Col-0, *sid2*, *jar1*, and *ein2* leaves inoculated with a suspension of *A. brassicicola* conidia deposited in the center of the leaves. Treatment with AgN23 was performed by spraying a bacterial suspension from a 7-day-old culture on the surface of the leaves. For each treatment, leaves were subjected to Trypan Blue staining to visualize necrotic lesions. Scale bar = 1 cm.

Materials were examined microscopically at an accelerating voltage of 5.00 kV.

To detect necrotic areas in *Arabidopsis* leaves, we used a Trypan staining solution containing 10 g of phenol, 10 ml of glycerol, 10 ml of lactic acid, 10 ml of ultrahigh-quality water, and 0.02 g of Trypan Blue in 60 ml of pure ethanol. Leaf material was placed in a 2-ml tube with Trypan Blue solution, boiled for 1 min, and left overnight in the staining solution at 20°C. The samples were washed two times in chloral hydrate destaining solution (1 kg of chloral hydrate in 400 ml of water).

SA quantification.

Determination of SA content was performed according to Vergnes et al. (2014). Briefly, 100 mg of leaf tissue was ground in liquid nitrogen, and 50 ng of an internal standard (o-anisic acid [oANI]) was added before the extraction of total SA. Total SA (free SA and SA conjugates) was extracted by methanolic extraction followed by acid hydrolysis. The hydrolysate was then subjected to organic phase partitioning (ether). Organic phase was evaporated and the sample diluted with 100 µl of acetonitrile-water-formic acid (50:50:0.1% [vol/vol/vol]). Analysis was performed with reverse-phase high-performance liquid chromatography (Ultimate 3000; Thermo Scientific) coupled with fluorescence detection (Jasco FP-920). SA and oANI were separated on an XBridge reverse-phase column (25 cm by 4.6 mm by 5 µm; Waters) and an XBridge guard column (2 cm by 4.6 mm by 5 µm; Waters) by gradient elution with a binary system of acetonitrile-water-formic acid (100:0.1% [vol/vol]) and water-formic acid (100:0.1% [vol/vol]) at a flow rate of 0.8 ml min⁻¹. Fluorescence detection set at excitation and emission wavelengths of 294 and 359 nm, respectively, for oANI and 305 and 407 nm for SA. SA was quantified using Chromeleon 6.8 chromatography software (Thermo Scientific). Corrections for losses were made for each individual sample according to recoveries of the internal standard.

Statistics.

Depending on the number of variables, one-way or two-way analysis of variance followed by Tukey's or Dunnett's multiple comparisons tests were performed after checking the

assumptions of normality by the Shapiro-Wilk test and equality of variance by the Brown-Forsythe test using GraphPad Prism version 7.05 for Windows. The test used for each dataset is stated in the figure legends.

Accession numbers.

Datasets generated or analyzed during this study are included in this published article (and its Supplementary Dataset files). Raw data regarding RNAseq analysis can be found on the NCBI Gene Expression Omnibus (GSE119986). The *Streptomyces* sp. AgN23 16S rRNA gene partial sequence was deposited on GenBank under the accession JN104730.1.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Empirical analysis of DGE: http://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/750/index.php?manual=Empirical_analysis_DGE.html

Corrected *P* values: http://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/900/index.php?manual=Corrected_p_values.html

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Fig. 8. Sporulation of *Alternaria brassicicola* on *Arabidopsis* leaves 6 days after inoculation. Leaves were treated with water or AgN23 48 h before inoculation. Error bars represent standard error of the mean of *n* = 3 independent experiments; differences in spore counts were assessed using two-way analysis of variance followed by Dunnett's multiple comparison test (** indicates *P* < 0.01).

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