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Characterization of the incompatible interaction between *Erwinia tracheiphila* and non-host tobacco (*Nicotiana tabacum*)

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ABSTRACT

Bacterial wilt is one of the most destructive diseases affecting a wide range of crops in the Cucurbits family including muskmelon (*Cucumis melo*), cucumber (*Cucumis sativus*), and squash (*Cucubita pepo*). The disease is caused by *Erwinia tracheiphila*, a Gram-negative and xylem-inhabiting species of *Erwinia*, which pathogenic mechanism is poorly understood. Many Gram-negative phytobacteria induce hypersensitive response (HR) in non-host plants, an immunity reaction triggered by pathogen recognition. With some exemptions, *Erwinia* species—notably *E. amylovora*, the causative agent of fire blight of rosaceous crops, and the reclassified soft rot pathogens, *Pectobacterium* and *Dickeya* species (formerly *E. carotovora* and *E. chrysanthemi*)—have been known to elicit HR in tobacco. However, concerning its pathogenic mechanism, the elicitation of classic HR has not been reported for some less-studied *Erwinia* species including *E. tracheiphila*. We characterized the induction of HR by the bacterial wilt pathogen in tobacco (*Nicotiana tabacum* cultivar 'Xanthi') using visual and physiological methods. We surveyed 21 *E. tracheiphila* strains and found that all of them elicited programmed cell death. Three strains (HCa1-5, UnisCu1-1, and MISpSq) fluorescently labeled with GFP could be visualized in the infiltrated leaves. We aligned the sequences of their HR-inducing protein, harpin (HrpN), predicted the secondary structures, and located the position of putative HR elicitors. We discovered differences between *Cucurbita* and *Cucumis* strains and found a close association of *E. tracheiphila* HrpN with those of *Pantoea* sp., *Erwinia piriflorinigrans*, and *Erwinia pyrifoliae*. Pre-infiltration of tobacco leaves with a lower cell population prevented HR following a subsequent challenge at the same area with HR-inducing levels of inoculum. The selected strains induced leaf conductivity levels similar to the HR-inducing *E. amylovora* strain E9, and their populations in the leaves decreased days after infiltration. Our results indicate that *E. tracheiphila* induces a classic HR in tobacco just like other HR-inducing *Erwinia* species.

1. Introduction

Bacterial wilt of cucurbits is one of the most destructive diseases of susceptible cucurbit species [1]. The disease is caused by an insect-transmitted, xylem-dwelling Enterobacterial phytopathogen, *Erwinia tracheiphila*. Despite its economic importance, the pathogen is one of the least studied *Erwinia* species—partly due to the success and popularity of insecticide-based bacterial wilt disease management and the difficulty of culturing the bacterium *in vitro* [2]. Since very little is known about this pathogen, there is a lot that the scientific community needs to know about not only its basic biology but also the various mechanisms of interaction with both compatible and incompatible host plants.

When a host plant is exposed to a pathogen, their interaction has one of two outcomes: disease or host resistance. A compatible interaction leads to disease while an incompatible one induces innate immunity from the plant. The two broadly defined and sometimes overlapping mechanisms of host defense against pathogens are pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) [3]. Defined molecular motifs (PAMPs) such as flagellin, lipopolysaccharide, and peptidoglycan are signature pathogen molecules that interact with the surface of plant cells and induce PTI [4–6]. When the plant detects bacterial effectors—proteins that are directly injected into plant cells through the type III secretion system—effector-triggered immunity (ETI) is elicited [7]. A compatible

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interaction leading to disease occurs in the absence of the plant resistance (*R*) gene that recognizes the effector avirulence (*avr*) gene product from the pathogen, while resistance happens when the plant triggers programmed cell death through hypersensitive response (HR) as a result of pathogen recognition [5,8].

Many Gram-negative bacteria induce HR in non-host plants [9]. The visual characteristics of the response include a rapid and programmed death of few host cells (apoptosis) that prevent the spread of the pathogen. Physiologically, reactive oxygen intermediates and ion fluxes in the cells are observed during the process [10–13]. The first reported pathogen-associated cell-free HR elicitor (harpin) gene, *hrpN*, was cloned from *Erwinia amylovora* [14]. Harpins are glycine-rich, heat-stable proteins, which are reported to act both as PAMPs (targeted extracellular of plant cells and functions as translocators) and effectors to elicit plant immunity [3,4,15,16]. Although the *hrpN* genes from some erwiniae including *E. amylovora, Dickeya dadantii* (*E. chrysanthemi*), and *Pantoea stewartii* (*E. stewartii*) code for HR elicitors [17–19], some others including *E. billingae* and *Pectobacterium carotovorum* (*E. carotovora* subsp. *carotovora*) lack the ability to induce HR in the wild type [20,21]. However, gene knockout of a global regulator, *rsmA*, resulting in a de-repressed expression of *hrpN*, enabled *P. carotovorum* subsp. *carotovorum* to elicit HR in tobacco [20].

Because *E. tracheiphila* has received little research attention, we have insufficient knowledge about its mechanisms of interaction with its host(s). However, the pathogen shares some features with other species of *Erwinia* or *Pantoea*, which can be used to understand these mechanisms. First, *E. tracheiphila* produces copious amount of extracellular polysaccharide like *E. amylovora* and *P. stewartii* and demonstrates motility through the action of peritrichous flagella [22]. Secondly, *E. tracheiphila* induces wilting symptoms on the host resembling those caused by *E. amylovora* on rosaceous species, especially at the onset of the infection. These similarities have led us to hypothesize that *E. tracheiphila* also possesses some of the same mechanisms of host-microbe interactions with other erwiniae. Particularly, we believe that just like the other HR-inducing erwiniae, *E. tracheiphila* has a functional *hrpN*, whose product, HrpN, will induce HR in a non-host plant. Our goals here are to gather evidence that *E. tracheiphila* induces classic HR in tobacco, compare the HrpN sequences of different strains of the pathogen, and determine the phylogenetic relationships of selected strains with the other members of Enterobacteriaceae based on HrpN.

2. Materials and methods

2.1. Media and bacterial and plant growth conditions

Bacterial strains (Table 1) were grown on Nutrient Broth-Yeast Extract (NY) plates at 28 °C for 3–4 days as previously described [22]. When needed, antibiotics were supplemented to the media at the indicated rates, nalidixic acid (Nal) at 25 μg/ml and ampicillin (Ap) at 100 μg/ml). Tobacco cultivar 'Xanthi' seeds were sown in a tray with Fafard Mix No. 2 (Sun Gro Horticulture, MA, USA). One week after germination, the plants were transplanted to 6-inch diameter pots (Grower's Solution LLC, Cookeville, TN, USA) and grown in a 14/10-h light/dark cycle. Two weeks after transplanting, plants were fertilized with complete fertilizer (14-14-14) at a rate of 2 g/pot. The plants used in the experiments were at five-to six-leaf stage.

2.2. HR survey among E. tracheiphila strains

Twenty-one *E. tracheiphila* strains isolated from cucumber (*Cucumis sativus*), melon (*Cucumis melo*), and squash (*Cucurbita pepo*) were surveyed for elicitation of HR in tobacco. The strains were streaked on NY agar media and the growth on each plate was scraped and resuspended in 2 ml sterile distilled water. The cell densities were standardized at 0.5 (5×10^6 CFU/ml) with A_{600} readings using a microplate reader (Synergy H1, Biotek, VT, USA). Sterile needleless 1 ml syringes (Henke Sass Wolf GmbH, Germany) were used to infiltrate the bacterial suspension in the apoplastic region of pre-pricked tobacco leaves. Symptoms of HR were observed 24 h after infiltration. The experiment was conducted twice in four replicates.

2.3. Construction of GFP plasmid

The plasmid pCKD300 was constructed by amplifying the promoterless *gfp* + sequence (~1000 bp) from pRU1701 [23] using *Taq* polymerase and primers Pac1_GFP + P6 (GAC*TTAATTAA*GCGGATTTGTCCTACTCAGG) and NotI_GFP + P5 (CTA*GCGGCCGC*TCACAAAACGGTTTACAAGCA). The following PCR parameters were used: 95 °C for 5 min, 1: 95 °C for 30 s, 2: 55 °C for 30 s, 3: 72 °C for 1 min × 30 cycles. The *gfp* + fragment was then ligated downstream of the *lac* promoter of plasmid pGEM®T-Easy (Promega, Madison, WI). The ligation reaction was electroporated into *E. coli* XL 10 Gold and plated on LB Agar supplemented with ampicillin. A bright fluorescent colony was selected in which the *gfp* + gene is under the control of the lac promoter in the multiple cloning site of the vector pGEMT-Easy. The DNA of pCKD300 was purified using Wizard® Plus SV Minipreps (Promega, Madison, WI).

2.4. GFP detection in planta

The selected strains, HCa1-5N and UnisCu1-1N, (*Cucumis* isolates), and MISpSq-N (*Cucurbita* isolate) were labeled with green fluorescent protein (GFP) by transformation with the plasmid pCKD300. Transformants were selected in LB + Ap and GFP was observed using a handheld UV lamp (365 nm, UVP, Upland, CA, USA). In a separate study, we determined that pGEM-T Easy plasmid replicon has almost 100% stability over three overnight sub-culturings in the *E. tracheiphila* strains used in this study (Prestwich and Dumenyo, unpublished). The labeled strains were infiltrated in tobacco leaves using the method described above. After 24 h, leaves were exposed under UV radiation to visualize fluorescent bacteria in the infiltrated region.

2.5. Sequence alignment, protein prediction, and phylogenetic tree

The predicted harpin sequences of *E. tracheiphila* HCa1-5, UnisCu1-1, and MISpSq were pulled out from our draft sequences (unpublished data) and aligned with that of *E. tracheiphila* PSU-1 harpin (Genbank accession no. EOS94912.1) using CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo). The secondary structure of harpins were predicted using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred), amino acid and isoelectric point using ExPASy ProtParam tool (http://web.expasy.org/protparam), and putative HR elicitor using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle) alignment with *E. pyrifoliae* HR elicitor. Multiple sequence alignment of the harpin proteins of *E. tracheiphila* and other Enterobacteriaceae was conducted using COBALT (http://www.st-va.ncbi.nlm.nih.gov/tools/cobalt). The alignment was downloaded as a Newick file and then uploaded to iTOL (http://itol.embl.de) to generate the phylogenetic tree. All software analyses were carried out in default parameters.

2.6. Conductivity test

The procedures for the conductivity test were modified from