Zucchini Vein Clearing Disease Is Caused by Several Lineages Within *Pseudomonas syringae* Species Complex

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ABSTRACT

Zucchini (*Cucurbita pepo*) is worldwide affected by *Pseudomonas syringae*, inducing vein clearing, stunting, and necroses during plantlet development. A collection of 58 *P. syringae* strains isolated from diseased zucchini plantlets was characterized by multilocus sequence analysis (MLSA). A subset of 23 strains responsible for vein clearing of zucchini (VCZ) was evaluated for pathogenicity on zucchini, and their genomes were sequenced. The host range of six VCZ strains was evaluated on 11 cucurbit species. Most VCZ strains belong to clades 2a and 2b-a within phylogroup 2 of *P. syringae* species complex and are closely related to other strains previously isolated from cucurbits. Genome analyses revealed diversity among VCZ strains within each clade. One main cluster, once referred to by the invalid pathovar name (*peponis*), gathers VCZ strains presenting a narrow host range including zucchini and squashes. Other VCZ

strains present a large host range including zucchini, squashes, cucumber, melons, and in some cases watermelon. The VCZ strain pathogenic features are strongly associated with type III effector repertoires. The presence of *avrRpt2* and absence of *hopZ5* are associated with a narrow host range, whereas the presence of *hopZ5* and absence of *avrRpt2* are most generally associated with a large host range. To better detect the different clusters identified with whole genome sequence and pathogenicity analyses, we used a specific-*k*-mers approach to refine the MLSA scheme. Using this novel MLSA scheme to type *P. syringae* isolates from diseased cucurbits would give insight into distribution of worldwide strains and origin of epidemics.

Keywords: bacterial seedborne disease, *Cucurbita pepo*, cucurbits, ecology and epidemiology, etiology, *Pseudomonas syringae*

In 2004 diseased plantlets of zucchini (*Cucurbita pepo* subsp. *pepo*) were observed in plant growers' facilities in Europe. Diseased plants show cotyledon and leaf necroses, vein clearing, and stunting. This disease is observed on plantlets and affects plant development at early stages, but to date no symptoms have been observed on fruits. One causal agent has been identified as belonging to the *Pseudomonas syringae* species complex and was provisionally named *P. syringae* pv. *peponis* (Manceau et al. 2011). This pathogenic bacterium was shown to be seed-transmitted (Manceau et al. 2011). Climatic conditions drastically influence symptom expression at early stage development; for example, low temperatures are associated with greater disease severity. Because most generally efficient chemical options are not available for bacterial threats, more knowledge about *P. syringae* responsible for vein clearing on zucchini (VCZ) is essential to implement appropriate control strategies.

The *P. syringae* species complex comprises 19 phylogenomic species based on average nucleotide identity (ANI) (Gomila et al. 2017), some of which are formally described (*P. amygdali*, *P. asturiensis*, *P. avellanae*, *P. cannabina*, *P. caricapapayae*, *P. caspiana*, *P. cerasi*, *P. cichorii*, *P. congelans*, *P. syringae*, and *P. viridiflava*). Moreover, *P. coronafaciens* has been proposed recently by Dutta et al. (2018). However, some of the crop pathogenic species are not easy to refer to because recombination between lineages is sufficiently high to blur the taxonomic boundaries (Baltrus et al. 2017; Dillon et al. 2019a). Many studies

devoted to classifying pathogenic strains of *P. syringae sensu lato* refer to phylogroups. Thirteen phylogroups (i.e., some phylogroups cluster several phylogenomic species) generally subdivided into clades were defined in the *P. syringae* species complex based on multilocus sequence analysis (MLSA) (Berge et al. 2014), and >60 pathovars were described based on pathogenicity (Young 2010). Indeed, a pathovar is defined as a group of strains showing the same or similar characteristics at an infra-subspecific level but presenting a distinctive pathogenicity, which means a unique host range or unique symptomatology (Dye et al. 1980).

Various pathogenic *P. syringae sensu lato* have been reported on cucurbits. *P. amygdali* pv. *lachrymans* causes angular leaf spots on cucumber (Bradbury 1986) and other cucurbits (Hopkins and Schenck 1972; Shila et al. 2013). Other strains of *P. syringae* that were named pathovar *lachrymans* cause different symptoms on cucumber and belong to the phylogenomic species *P. tomato* within the phylogroup 1 (Gomila et al. 2017; Słomnicka et al. 2018). Diverse strains isolated from melons and squashes within phylogroup 2 belong to *P. syringae* pv. *aptata*, which was initially described as pathogenic on sugar beet (Morris et al. 2000; Sedighian et al. 2014). Other strains of *P. syringae sensu lato* genetically different from the two previously mentioned pathovars were shown to induce bacterial leaf spots on adult watermelon, melon, and squash (Newberry et al. 2016, 2017, 2018, 2019; Tian et al. 2017) and bacterial warts on pumpkin fruits (Tyman and Inglis 2017). Most of these cucurbit pathogenic strains are phylogenetically diverse (Newberry et al. 2016, 2017, 2018, 2019). These strains belong to different species, including *P. cerasi* (Kałużna et al. 2016), which corresponds to phylogroup 2a (Berge et al. 2014); *P. syringae* (corresponding to phylogroup 2b, Berge et al. 2014); the unnamed group A *sensu* Gomila et al. (2017; also named phylogroup 2d, Berge et al. 2014); and the phylogroup 2b-a, issued from the recombination of *P. syringae* and *P. cerasi* (Newberry et al. 2019). In other words, these strains belong to different clades (i.e., 2a, 2b, 2b-a, and 2d) within the phylogroup 2 of the *P. syringae* species complex. The phylogroup 2 appears to be the most ubiquitous group and comprises

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numbers of pathogenic strains with narrow or wide host range but also strains recovered from the environment, pathogenic or not (Baltrus et al. 2017; Berge et al. 2014; Monteil et al. 2013; Morris et al. 2000).

Cucurbitaceae is a family of plants including economically important crops such as cucumbers (*Cucumis sativus* L.), melons (*Cucumis melo* L.), watermelons (*Citrullus lanatus*), and squashes. *Squash* is a generic name for various plant species including *Cucurbita pepo*, *Cucurbita maxima*, and *Cucurbita moschata*. Zucchini together with pumpkin, vegetable marrow, and cocozelle form *Cucurbita pepo* subsp. *pepo*. Acorn, scallop, straightneck, and crookneck form *Cucurbita pepo* subsp. *texana* (Paris 1989; Paris et al. 2015). Genetic relationships between many *Cucurbita pepo* were inferred from the calculation of average dissimilarity values using DNA markers (Paris et al. 2015). These results indicate that zucchini and pumpkin from the same subspecies are genetically close, whereas zucchini and straightneck are more distant.

Pathogenicity assays are essential to determine epidemic potential of phytopathogenic bacteria. Such assays constitute a prerequisite to designate pathovars distinguished by distinct host range and symptoms (Bull et al. 2008). Host range and virulence of *P. syringae sensu lato* are shaped by virulence factors such as type III effectors (T3Es) and phytotoxins (Silby et al. 2011; Xin et al. 2018). T3Es are secreted via the type III secretion system to specifically target host proteins or DNA sequences inside plant cells and alter physiological processes to the benefit of the pathogens (Feng and Zhou 2012). The *P. syringae* content of T3Es evolves, and T3Es can be acquired via horizontal transfer as response to host-mediated selection (Baltrus et al. 2011; Dillon et al. 2019a, b).

Several methods based on genomic DNA sequences are used to study organism phylogeny and update taxonomy. MLSA is very useful to assign strains to previously described taxa and reveal phylogenetic relationships between strains (Jacques et al. 2012, 2016, Young 2010). This portable and easy-to-use method is based on four or more housekeeping genes. Within the *P. syringae* species complex, a classic MLSA scheme initially proposed by Sarkar and Guttman (2004) and refined by Hwang et al. (2005) is often used to describe genetic structure of strain groups and to assign unknown strains to the correct genetic group and pathovar within the *P. syringae* species complex (Bull et al. 2011; Cuntz et al. 2015; Ferrante and Scortichini 2015; Martín-Sanz et al. 2013; Newberry et al. 2016; Słomnicka et al. 2015). Furthermore, several methods based on whole genome sequence comparisons are currently used to infer closely related bacteria relationships, such as the average nucleotide identity based on BLAST (ANiB) (Konstantinidis and Tiedje 2005). Other methods, based on the number of words of length *k* (*k*-mers) shared between genome sequences, could be used as proxy for species delimitation (Briand et al. 2019). In addition, distribution of *k*-mers in a specific genomic group could provide a list of markers for the identification of bacterial strains (Denancé et al. 2019).

To gain knowledge on bacteria causing VCZ and to define appropriate field management strategies, we developed a three-step approach. Our first aim was to correctly allocate the VCZ strains in the *P. syringae* species complex and infer how these strains are related to other *P. syringae* isolated from cucurbits with MLSA and a whole-genome studies. Second, different pathogenicity assays were designed to finely phenotype strains on their host of origin (zucchini) but also on diverse cucurbit genotypes. Third, based on draft genome sequences, some specific T3Es were highlighted for their potential link with host range specialization, and a new MLSA scheme dedicated to this group of pathogens was developed. This work allows better evaluation of the epidemic potential of VCZ strains.

MATERIALS AND METHODS

Bacterial strain collection. Fifty-eight strains were isolated from zucchini seedlings (*Cucurbita pepo* subsp. *pepo*) with symptoms

of vein clearing issued from seed lots harvested in different geographic locations between 2002 and 2015 (Table 1). Bacterial strains were stored in a -80°C freezer in 40% glycerol solution and were routinely cultivated at 28°C on 3 g/liter of tryptone soya broth, 15 g/liter of agar). All these strains induced typical VCZ symptoms (necrosis, vein clearing, and stunting) on zucchini after inoculation by rubbing the cotyledons.

Housekeeping gene sequencing. Amplicons were obtained from fresh pure cultures (overnight growth at 28°C on 3 g/liter of tryptone soya broth, 15 g/liter of agar) using primers developed by Hwang et al. (2005) for the four following housekeeping genes: *gapA* (encoding the glyceraldehyde-3-phosphate dehydrogenase A), *gltA* (encoding the citrate synthase), *gyrB* (encoding the B subunit of DNA gyrase), and *rpoD* (encoding the RNA polymerase sigma70 factor RpoD). Briefly, PCRs were performed in a 50- μl reaction mixture containing 0.2 μl of reaction buffer, 0.2 μM dNTP, 0.5 μM of each primer, and 10 μl of boiled bacterial cells. The amplification program consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C (*gapA*) or 64°C (*gltA*, *gyrB*, and *rpoD*) for 30 s, extension at 72°C for 1 min 30 s, and a final extension for 7 min at 72°C . Sanger sequencing was performed on forward and reverse strands.

Sequence acquisition and alignment. Forward and reverse nucleotide sequences were assembled and aligned according to the reading frame of a complete gene sequence and trimmed with Bionumerics, version 7.6 and Geneious, version 9.1.7. Sequences were trimmed to obtain fragments of 474 bp (*gapA*), 528 bp (*gltA*), 507 bp (*gyrB*), and 498 bp (*rpoD*) and eventually concatenated according to the alphabetical order in a 2,007-bp-long sequence.

MLSA. Phylogenetic analyses were first performed on individual partial gene sequences of *gapA*, *gltA*, *gyrB*, and *rpoD* and then on the concatenated dataset to obtain one single fragment of 2,007 bp. These sequences were analyzed together with publicly available sequences from 22 strains representing the main phylogroups and clades (Berge et al. 2014) of the *P. syringae* species complex and from 17 *P. syringae* strains (13-509A, Ps711, 13-140A, 13-C2, 14-32, 14-410, 14-Gil, Zum3584, 200-1, Bs2121, Zum3984, 03-19A, Bs13-139B, 13-429, Ps03-A13, PsBS3058, and Ps02-B3) isolated from various cucurbits (Newberry et al. 2016, 2019) (Supplementary Table S1). Sequences from strain CFBP 2107 (*P. viridiflava*) were used to root the trees.

For the novel MLSA scheme proposed in this study, the analysis of the four gene sequences previously mentioned (*gapA*, *gltA*, *gyrB*, and *rpoD*) and genes identified in this study *Psyr3420*, *Psyr4880*, and *Psyr3208* led to the acquisition of one unique fragment of 4,431 bp. Sequences were recovered from the genome sequences of a strain subset from our collection (23 VCZ strains), a subset of 14 strains isolated from cucurbits by Newberry et al. (2019), and 22 diverse strains from phylogroup 2 (Supplementary Table S1).

The Shimodaira-Hasegawa (SH) (Shimodaira and Hasegawa 1999) test implemented in the DnaML program from PHYLIP, Phylogeny Inference Package, version 3.69 (Felsenstein 1989) was used to test whether tree topologies fell within the same confidence limits. Maximum likelihood trees were generated with MEGA 7 (Kumar et al. 2016) via the Tamura-Nei model with gamma distributed with invariant sites (G + I) and 1,000 bootstrap replicates.

Genome sequencing. A subset of 23 VCZ strains were chosen to maximize their genetic diversity and their geographic origin (P99, P121, P123, P12855, P84, P12831, P139, P12857, P127, P79, P22, P12854, P12832, P113, P5, P118, P66, P77, P78, P87, P89, P73, and P108). The pathotype strain of *P. syringae* pv. *dysoxyl* (strain CFBP 2356) was also included in this study. Genomic DNAs of these strains were obtained using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The genomic DNA quality was checked

with NanoDrop ND-1000 spectrophotometer (the NanoDrop Technologies, Wilmington, DE) and quantified using a Qubit fluorometer (Invitrogen). Sequencing libraries were prepared using TruSeq DNA PCR-Free kits (Illumina). DNAs of P66 and P99 strains were paired-end sequenced (2×300 bp) using the Illumina MiSeq v3 (ANAN platform, SFR QuaSav, Angers, France). DNAs of the other VCZ strains and strain CFBP 2356 were paired-end sequenced (2×150 bp) using the Illumina HiSeq X-Ten (Beijing Genomics Institute, China).

Genome sequence analysis. Genome assembly was performed with SOAPdenovo version 2.04, SOAPGapCloser version 1.12 (Luo et al. 2012), and Velvet version 1.2.02. (Zerbino and

Birney 2008) Genome sequences were annotated with EuGene-PP (Sallet et al. 2014). A dataset of 116 genome sequences (Supplementary Table S1) composed of 23 genome sequences from strains responsible for VCZ, a collection of 14 genome sequences (13-509A, Ps711, 13-140A, 13-C2, 14-32, 14-410, 14-Gil, Zum3584, 200-1, Bs2121, Zum3984, 03-19A, Bs13-139B, and 13-429) of *P. syringae* isolated from cucurbits (Newberry et al. 2019), the genome sequence of CFBP 2356 (*P. syringae* pv. *dysoxyl*), and 78 genome sequences available in NCBI (to represent *P. syringae* diversity from phylogroup 2) was used for phylogenetic analyses. To assess relationships between genome sequences, the ANIb (Konstantinidis and Tiedje 2005) and percentage of shared 15-mers (Briand et al. 2019) were used. ANIb

TABLE 1. The 58 vein clearing of zucchini (VCZ) strains used in this study

VCZ strain number	Origin	Year	Host	Genome accession numbers
P1	France	2005	Zucchini	
P100	na ^a	2011	Zucchini	
P101	na	2011	Zucchini	
P102	na	2011	Zucchini	
P104	na	2011	Zucchini	
P108	Chile	2011	Zucchini	VLIA000000000
P112	Chile	2011	Zucchini	
P113	Thailand	2011	Zucchini	VLIC000000000
P115	China	2011	Zucchini	
P118	France	2013	Zucchini	VLID000000000
P119	France	2013	Zucchini	
P12	France	2006	Zucchini	
P120	France	2013	Zucchini	
P121	Chile	2013	Zucchini	VLIE000000000
P123	China	2013	Zucchini	VLIF000000000
P125	China	2013	Zucchini	
P127	India	2013	Zucchini	VLIG000000000
P12831	France	2005	Zucchini	WJPZ000000000
P12832	France	2006	Zucchini	VLHX000000000
P12833	France	2005	Zucchini	
P12836	France	2005	Zucchini	
P12854	France	2002	Zucchini	VLHY000000000
P12855	Chile	2007	Zucchini	VLHZ000000000
P12856	France	2005	Zucchini	
P12857	France	2007	Zucchini	VLIB000000000
P130	Thailand	2013	Zucchini	
P131	China	2013	Zucchini	
P133	Thailand	2013	zucchini	
P136	India	2014	Zucchini	
P137	France	2014	Zucchini	
P138	France	2015	Zucchini	
P139	China	2015	Zucchini	VLIH000000000
P14	na	2008	Zucchini	
P22	USA	2009	zucchini	WJPQ000000000
P24	France	2008	Zucchini	
P4	France	2005	Zucchini	
P5	France	2008	Zucchini	WJPR000000000
P55	Chile	2009	Zucchini	
P56	na	2009	Zucchini	
P62	France	2010	Zucchini	
P65	na	2010	Zucchini	
P66 (CFBP 8692)	Thailand	2010	Zucchini	WJSF000000000
P68	USA	2010	Zucchini	
P69	USA	2010	Zucchini	
P70	Thailand	2010	Zucchini	
P71	na	2010	Zucchini	
P72	na	2010	Zucchini	
P73	USA	2010	Zucchini	WJPS000000000
P77	France	2010	Zucchini	WJPT000000000
P78	France	2010	Zucchini	WJPU000000000
P79	France	2010	Zucchini	WJPV000000000
P84	China	2010	Zucchini	WJPW000000000
P87	China	2011	Zucchini	WJPX000000000
P88	China	2011	Zucchini	
P89	France	2011	Zucchini	WJPY000000000
P90	USA	2011	Zucchini	
P94	na	2011	Zucchini	
P99 (CFBP 8693)	France	2005	Zucchini	WJSE000000000

^a na, not available.

values were obtained with the online version of pyani (<https://github.com/widowquinn/pyani>), and percentage of shared *k*-mers and distance matrices were calculated with Ki-S, as were dendrograms (<https://iris.angers.inra.fr/galaxypub-cfbp>) (Briand et al. 2019). Specific *k*-mers in predicted coding sequences (CDSs) of each genomic group were identified with SkIf (Denancé et al. 2019). SkIf was also used to identify sequences shared by all VCZ strains and absent in the predicted proteins of other closely related pathovars. Homology groups were identified in predicted proteomes by an OrthoMCL analysis (80% identity on 80% length) using the Family-Companion tool (<http://family-companion.toulouse.inrae.fr/>) (Cottret et al. 2018). Finally, the T3E repertoire of each strain was predicted through tBLASTN search (identity >80% on ≥80% of CDS length) on 138 amino acid sequences of T3Es (Hops database at <http://www.pseudomonas-syringae.org/>). Putative frameshift mutations were checked by aligning each T3E sequence with the corresponding curated reference sequence with MultAlin (<http://multalin.toulouse.inra.fr/multalin/>) (Corpet et al. 1999).

Pathogenicity tests on zucchini. To characterize the pathogenicity of strains on their host of origin (zucchini), a cotyledon rubbing test and two seed soaking tests were run on *Cucurbita pepo* subsp. *pepo* ‘Tosca’ and ‘Opale’. For each test, 23 strains responsible for VCZ (P99, P121, P123, P12855, P84, P12831, P139, P12857, P127, P79, P22, P12854, P12832, P113, P5, P118, P66, P77, P78, P87, P89, P73, and P108), and four controls were inoculated. Water was used as negative control, CFBP 5427 (*P. syringae* pv. *aptata*) and CFBP 6463 (*P. amygdali* pv. *lachrymans*) were used as pathogenic controls on zucchini, and CFBP 2356 (*P. syringae* pv. *dysoxyl*), which is a woody plant pathogen, was used as negative control.

In the cotyledon rubbing test, five plantlets at the cotyledon stage were inoculated per strain and cultivar by rubbing the cotyledon surface three times with a finger soaked in bacterial suspensions calibrated at 1×10^8 cfu/ml. Plantlets were produced in a greenhouse from healthy seeds for 7 days in pots containing Klassman substrate and watered as needed for optimal growth. Inoculated plantlets were incubated in a growth chamber (18°C night, 25°C day, 14 h daylight, relative humidity near 100%), and the health status of each plantlet was recorded 8 days postinoculation (dpi). Plantlets were qualitatively rated, and the number of plantlets with necrosis on leaves, vein clearing on leaves, and stunting was counted.

For the two seed soaking tests, 10 seeds per strain and per zucchini cultivar (*Cucurbita pepo* subsp. *pepo* ‘Tosca’ and ‘Opale’) were inoculated by a 30 min soaking in a fresh bacterial suspension calibrated at 1×10^6 cfu/ml or 1×10^7 cfu/ml. Then, seeds were dried under a laminar flow in individual Petri dishes containing filter paper for 2 h. Each dried seed was sown in an individual small pot containing Klassman substrate and was watered. Pots were incubated in a climatic chamber (18°C night, 25°C day, 14 h daylight, relative humidity near 100%). Health status of each plantlet was evaluated 8 days after sowing for both inoculum doses and ≤14 days for test with a 1×10^7 cfu/ml inoculum concentration. Plantlets were considered healthy when no macroscopic symptom was visible and diseased when plantlets presented at least one of the following symptoms: necrosis on cotyledons, vein clearing of the first leaf, or stunting.

Pathogenicity tests on different cucurbit species. To evaluate the host range of the VCZ strains, pathogenicity tests were run on a collection of 11 cucurbit genotypes. Six squashes including zucchini (*Cucurbita pepo* subsp. *pepo* ‘Opale’ and ‘Tosca’), a straightneck squash (*Cucurbita pepo* subsp. *texana* ‘Cheetah’), a pumpkin (*Cucurbita pepo* subsp. *pepo* ‘Mischief’), a French pumpkin (*Cucurbita maxima* ‘Rouge Vif d’Etampes’), and a butternut (*Cucurbita moschata* ‘Sibelle’) were tested. Three melon varieties including a cantaloupe (*Cucumis melo* var. *cantalupis* ‘Perseus’), a Galia melon (*Cucumis melo* var. *galia* ‘Citrex’), and a Canary melon (*Cucumis melo* var. *inodorus* ‘Helios’); a cucumber (*Cucumis sativus* ‘Dahab’); and a watermelon (*Cucumis lanatus*

‘Livia’) were tested. Seeds were sown individually in pots containing Klassman substrate and germinated in a greenhouse until the first true leaf stage. The day before inoculation, plantlets were moved for acclimation to inoculation cages in growth chamber (18°C night, 25°C day for 14 h). Each inoculation cage contained five plantlets of each cucurbit genotype. Six strains responsible for VCZ (P99, P79, P66, P73, P77, and P108) and three controls including water, CFBP 5427 (*P. syringae* pv. *aptata*), and CFBP 6463 (*P. amygdali* pv. *lachrymans*) were evaluated. The assay was independently done twice for each genotype (except for *Cucurbita pepo* subsp. *texana* ‘Cheetah’). Bacterial suspensions were prepared from fresh cultures and calibrated at 1×10^7 cfu/ml. Each strain was sprayed until runoff on each plantlet. Plantlets were incubated for 7 days (18°C night, 25°C day for 14 h and near 100% relative humidity). Pathogenicity was qualitatively rated as (0) when a plantlet was asymptomatic and (1) when plantlets presented necrosis. First leaves were collected for photography on a light table.

Test of hypersensitive response. Ability of strains P99, P66, P108, and P12857 to elicit a hypersensitive response (HR) was tested on melons (*Cucumis melo* var. *galia* ‘Citrex’, *Cucumis melo* var. *cantalupis* ‘Perseus’, *Cucumis melo* var. *inodorus* ‘Helios’), cucumber (*Cucumis sativus* ‘Dahab’), and watermelon (*Citrullus lanatus* ‘Livia’). Additionally, strains P79, P77, and P73 were tested on watermelon. These strains were chosen based on phylogenetic position and to represent different pathogenicity groups. Pathogenicity controls were CFBP 5427 (*P. syringae* pv. *aptata*) on melons, CFBP 6463 (*P. amygdali* pv. *lachrymans*) on cucumber, and CFBP 4459 (*Acidovorax citrulli*) on watermelon. HR control was given by strain CFBP 2356 (*P. syringae* pv. *dysoxyl*) because this strain is nonpathogenic on cucurbits. Water was used as a negative control. At the time of inoculation, fresh inocula calibrated at 1×10^8 cfu/ml were infiltrated with a needle in the limb of the second leaf of 3-week-old plantlets produced in Klassman substrate in a greenhouse. The HR characteristics (rapid death of cells in the infiltration area) were visually evaluated at 48 and 72 h after inoculation on each inoculated leaf. Dried brown necrosis limited to the infiltrated area corresponded to HR, whereas spreading lesions that eventually collapsed with a water-soaked aspect corresponded to disease.

Nucleotide sequence accession numbers. The GenBank accession numbers for the partial sequences used in this study are MN176747 to MN176804 for *gapA*, MN176805 to MN176862 for *gltA*, MN176863 to MN176920 for *gyrB*, and MN176921 to MN176978 for *rpoD*. Genomic sequences of VCZ strains sequenced in this study were deposited in NCBI; accession numbers are listed in Table 1. The genomic sequence of *P. syringae* pv. *dysoxyl* CFBP 2356 sequenced in this study was deposited in NCBI (accession number VLHW00000000).

RESULTS

Phylogenetic analyses of VCZ strains. All strains responsible for VCZ belonged to phylogroup 2 of the *P. syringae* species complex (Fig. 1A). The maximum likelihood tree clearly showed that all VCZ strains grouped in the same branch, as did type strains and pathotype strains of phylogroup 2 (Fig. 1A). Other strains of *P. syringae sensu lato* isolated from various cucurbits (Newberry et al. 2016, 2019) also grouped in the same cluster. VCZ strains were genetically different from strain NM002 of *P. amygdali* pv. *lachrymans*, which is a pathovar previously reported as pathogenic on various cucurbits (Bradbury 1986) and belonging to phylogroup 3 (Słomnicka et al. 2018).

Within phylogroup 2, VCZ strains were assigned mainly to clade 2b-a and to clade 2a of the *P. syringae* species complex (Fig. 1B). Thirty-nine VCZ strains grouped in one highly supported cluster (bootstrap value of 100%) corresponding to clade 2b-a previously described by Newberry et al. (2019). This cluster also gathered eight

strains isolated from squash and watermelon (Newberry et al. 2019) and *P. syringae* pv. *syringae* HS191 isolated from millet (*Panicum miliaceum*). The closest type and pathotype strains to clade 2b-a were *P. syringae* pv. *syringae* CFBP 1392 and *P. syringae* pv. *aptata* CFBP 1617, isolated from lilac (*Syringa vulgaris*) and sugar beet (*Beta vulgaris*), respectively. A second highly supported cluster

(bootstrap value of 99%), previously described as clade 2a (Berge et al. 2014), grouped 15 VCZ strains and four strains isolated from cantaloupe, squash, and watermelon (Newberry et al. 2019). The closest type and pathotype strains were *P. syringae* pv. *dysoxylis* CFBP 2356, *P. syringae* pv. *syringae* cit7 and *P. syringae* pv. *papulans* LMG 5076 isolated from kohekohe (*Dysoxylum spectabile*), orange

Fig. 1. Phylogenetic relationships of strains responsible for vein clearing of zucchini (VCZ). The diversity of 58 VCZ strains and 17 strains isolated from diverse cucurbit species (Newberry et al. 2016, 2019) was assessed through multilocus sequence analysis. Maximum likelihood (ML) trees were based on concatenated partial sequences of *gapA*, *gltA*, *gyrB*, and *rpoD* (2,007 bp). Bootstrap scores (1,000 replicates) are displayed at each node. **A**, ML tree of typed strains representing the *P. syringae* group. Phylogroups (PGs) are derived from Bull and Koike (2015). **B**, Detail of phylogroup 2 diversity including VCZ strains. Clades are indicated based on Berge et al. 2014, Bull and Koike 2015, and Newberry et al. 2019. Black points indicate VCZ strains.

(*Citrus sinensis*), and apple tree (*Malus domestica*), respectively. The last four VCZ strains (P136_{2b-S}, P138_{2b-S}, P108_{2b-S}, and P101_{2b-S}) grouped in clade 2b but were clearly separated from clade 2b-a. Three strains isolated from watermelon (Newberry et al. 2016) were closely related to these four singletons.

According to the SH test, maximum likelihood trees performed on individual gene and the concatenated four genes were not congruent, suggesting recombination events among loci (Supplementary Fig. S1). Specifically, trees built on *gapA*, *gltA*, and *rpoD* were not congruent with the tree based on the concatenated dataset. The differences were limited between *gltA* and the concatenated dataset trees, whereas clusters behaved substantially differently when *gapA* or *rpoD* were analyzed separately.

Pathogenicity of VCZ strains on zucchini. All VCZ strains, *P. amygdali* pv. *lachrymans* CFBP 6463, and *P. syringae* pv. *aptata* CFBP 5427 were pathogenic on zucchini, as demonstrated with the cotyledon rubbing test, but only VCZ strains induced severe stunting (Fig. 2, Supplementary Table S2). More specially, 23 VCZ strains were evaluated on two zucchini cultivars (*Cucurbita pepo* subsp. *pepo* ‘Tosca’ and ‘Opale’). All VCZ strains induced cotyledon

necrosis and leaf necrosis on plantlets with little variability among the tested strains (Fig. 2). Short-lived vein clearing symptom was observed in the days after the inoculation of all VCZ strains, except for strains P22_{2ba-A} and P79_{2ba-A}, which did not induce vein clearing on one or the other cultivar (Supplementary Table S2). Most VCZ strains induced severe stunting except strains P22_{2ba-A} and P79_{2ba-A}, which were less aggressive than other strains. Strains *P. syringae* pv. *aptata* CFBP 5427 and *P. amygdali* pv. *lachrymans* CFBP 6463, previously reported as pathogenic on various cucurbits (Bradbury 1986; Morris et al. 2000), were used as controls. Both strains induced necroses on both cultivars that were similar to those induced by VCZ strains. Moreover, *P. syringae* pv. *aptata* CFBP 5427 was able to produce short-lived vein clearing on zucchini ‘Opale’ but not *P. amygdali* pv. *lachrymans* CFBP 6463. Neither CFBP 5427 nor CFBP 6463 was able to induce severe stunting on inoculated zucchini. No symptom was observed on plantlets inoculated with water and *P. syringae* pv. *dysoxyl* CFBP 2356, which is a pathotype strain from clade 2a that is not pathogenic on cucurbits.

Seed soaking tests revealed different levels of aggressiveness for VCZ strains, and overall, no differences in terms of aggressiveness

Fig. 2. Responses of two cultivars of zucchini to seed inoculation and cotyledon rubbing assay with *Pseudomonas syringae* strains. *Cucurbita pepo* subsp. *pepo* ‘Opale’ (orange) and ‘Tosca’ (blue) plants or seeds were inoculated with 23 strains of *P. syringae* responsible for vein clearing of zucchini (VCZ) and four control strains including CFBP 5427 (*P. syringae* pv. *aptata*), CFBP 6463 (*Pseudomonas amygdali* pv. *lachrymans*), CFBP 2356 (*P. syringae* pv. *dysoxyl*), and water. Bars indicate the percentage of disease incidence over the number of germinated plantlets 7 days after inoculation for a given strain and a given test (cotyledon rubbing, number of tested plantlets $n = 5$; seed soaking at 1×10^7 cfu/mL, number of sown seeds $n = 10$; seed soaking at 1×10^6 cfu/mL, number of sown seeds $n = 10$). SS*, seed soaking test.

on zucchini were linked to the genetic group of the strains (Fig. 2). With the seed soaking test in a suspension at 1×10^6 cfu/ml, VCZ strains were highly aggressive on ‘Opale’ and weakly aggressive on ‘Tosca’ (Fig. 2). With the seed soaking test in a higher concentrated suspension (1×10^7 cfu/ml), VCZ strains were highly aggressive on both cultivars, inducing necrosis on almost all plantlets. Strain P22_{2ba-A} was the only VCZ strain that did not induce any stunting with this test (Supplementary Table S2). *P. amygdali* pv. *lachrymans* CFBP 6463 and *P. syringae* pv. *aptata* CFBP 5427 induced limited necroses on a few plantlets in these seed soaking tests. No symptoms were observed on plantlets issued from seeds inoculated with water and *P. syringae* pv. *dysoxyl* CFBP 2356.

Host range of VCZ strains. All VCZ strains induced similar symptoms on zucchini and pumpkin (*Cucurbita pepo* subsp. *pepo*) cultivars (Fig. 3A). On zucchini (*Cucurbita pepo* subsp. *pepo* ‘Tosca’ and ‘Opale’) and on pumpkin (*Cucurbita pepo* subsp. *pepo* ‘Mischief’), the strains responsible for VCZ P99_{2ba-A}, P79_{2ba-A},

P66_{2a-E}, P77_{2a-D}, P73_{2a-D}, and P108_{2b-S} induced water-soaking spots evolving in necrotic spots surrounded by a yellow halo at 7 dpi. Interestingly, *P. syringae* pv. *aptata* CFBP 5427 also induced this type of symptom on these genotypes and could not be distinguished from the VCZ strains, whereas *P. amygdali* pv. *lachrymans* CFBP 6463 induced smaller irregular necrotic lesions.

All VCZ strains were pathogenic on straightneck squash (*Cucurbita pepo* subsp. *texana*) and winter squashes (*Cucurbita maxima*, *Cucurbita moschata*), but different types of symptoms were observed according to the MLSA group (Fig. 3A). The yellow straightneck squash (*Cucurbita pepo* subsp. *texana* ‘Cheetah’) seemed to be less susceptible to VCZ strains from clade 2b-a (P99_{2ba-A}, P79_{2ba-A}), which induced dried brown spots, whereas VCZ strains from clade 2a (P66_{2a-E}, P73_{2a-D}, P77_{2a-D}) induced water-soaked spots evolving in necrosis and were associated with chlorosis. On butternut (*Cucurbita moschata* ‘Sibelle’), VCZ strains from clade 2b-a induced brown necrotic spots with a yellow halo that collapsed, whereas strains from

Fig. 3. Host range test of vein clearing of zucchini (VCZ) strains. Six *Pseudomonas syringae* strains responsible for VCZ and three controls including water, *P. syringae* pv. *aptata* CFBP 5427, and *P. amygdali* pv. *lachrymans* CFBP 6463 were spray inoculated (1×10^7 cfu/ml until runoff) on six genotypes of *Cucurbita* sp., four genotypes of *Cucumis* sp., and one genotype of *Citrullus lanatus*. Water was sprayed as control. **A**, Photographs of the symptoms observed on the first leaf 7 days after inoculation. **B**, Results of pathogenicity test: +, disease symptoms; -, absence of symptoms.

clade 2a induced large water-soaking lesions and necrotic spots surrounded by a yellow halo. On *Cucurbita maxima* 'Rouge Vif d'Etampes', VCZ strains from clade 2b-a induced tiny yellow spots, whereas VCZ strains from clade 2a induced water-soaked spots surrounded by a yellow halo.

Only VCZ strains from clade 2a and the singleton P108_{2b-S} were pathogenic on the cucumber, melons, and watermelon cultivars that were tested (*Cucumis sativus*, *Cucumis melo*, *Citrullus lanatus*) (Fig. 3). VCZ strains from clade 2a (P66_{2a-E}, P73_{2a-D}, P77_{2a-D}) were pathogenic on all three melon varieties (*Cucumis melo* var. *galia* 'Citrex', *Cucumis melo* var. *inodorus* 'Helios', *Cucumis melo* var. *cantaloupe* 'Perseus') and induced necrotic spots surrounded by a yellow halo. On cucumber (*Cucumis sativus* 'Dahab'), VCZ strains from clade 2a were highly aggressive and induced collapsing circular necrotic spots with large chlorosis. In clade 2a, only strain P66_{2a-E} induced necrotic spots surrounded by a little yellow halo on watermelon (*Citrullus lanatus* 'Livia'). The tested cultivars of cucumber, melon, and watermelon were not susceptible to the VCZ strains from clade 2b-a (P99_{2ba-A}, P79_{2ba-A}). Some tiny dry white spots were sometimes visible on inoculated leaves, but plants remained healthy 2 weeks after inoculation. Singleton VCZ strain P108_{2b-S} was pathogenic on all tested cucurbits, but the symptoms were different from those caused by VCZ strains from clade 2a (P66_{2a-E}, P73_{2a-D}, P77_{2a-D}) on some hosts. For instance, on watermelon, P108_{2b-S} induced large dried necrotic spots, whereas P66_{2a-E} induced small spots with a discrete yellow halo. *P. amygdali* pv. *lachrymans* CFBP 6463 and *P. syringae* pv. *aptata* CFBP 5427 were pathogenic on almost all the tested genotypes, and the symptoms were sometimes different from those caused by VCZ strains. As expected, *P. amygdali* pv. *lachrymans* CFBP 6463 induced angular leaf spots on cucumber, and *P. syringae* pv. *aptata* CFBP 5427 induced circular necrotic spots on melons. All plantlets inoculated with water remained asymptomatic 7 dpi.

HR of different cucurbits toward VCZ strains. Infiltration tests on cucumber, watermelon, and melons confirmed that VCZ strains had different host ranges (Fig. 4). Starting from 48 h after inoculation, spreading lesions that eventually collapsed with a water-soaked aspect on the abaxial leaf surface were noticed for all pathogenic controls on their hosts (*A. citrulli* on watermelon, *P. amygdali* pv. *lachrymans* on cucumber, and *P. syringae* pv. *aptata* on melons). *P. syringae* pv. *dysoxyl* CFBP 2356, a nonpathogenic strain on cucurbits, induced HR with a dried brown necrosis limited to the infiltrated area. As expected, strains P66_{2a-E} and P108_{2b-S} caused water-soaked lesions similar to those of the corresponding pathogenic controls on cucumber and the three melon varieties. In contrast, strain P99_{2ba-A} caused the same HR-like necrosis as those of *P. syringae* pv. *dysoxyl* CFBP 2356 on all tested genotypes, confirming nonhost reaction. On watermelon, strain P66_{2a-E} was pathogenic, whereas strains P73_{2a-D} and P77_{2a-D} induced HR-like necrosis, confirming pathogenic heterogeneity on this host for strains of the clade 2a (Supplementary Fig. S2).

VCZ strains genome sequence analyses. Based on whole genome sequences, ANIb revealed strong relationships between VCZ strains from the 2a and 2b-a groups (Fig. 5, Supplementary Table S3). First, ANIb values between genome sequences of *P. syringae* strains from clades 2a and 2b (except 2b-a strains) were <95%, indicating that strains belonged to different species (Supplementary Table S3). Interestingly, the genome sequences from the 23 VCZ strains and the other 14 cucurbit strains (Newberry et al. 2019) included in clades 2b-a and 2a were more similar, with ANIb value >95%.

Based on whole genome sequences, new clusters inside the main genetic groups (clades 2b-a and 2a) were highlighted by ANIb and shared *k*-mer analyses (Fig. 5). Both dendrograms displayed similar phylogeny and showed three new clusters within each clade 2b-a and 2a (Fig. 5). Within clade 2b-a, the first cluster (A) gathered 15 VCZ strains and two cucurbit strains (Ps711_{2ba-A}, 13-509A_{2ba-A}) isolated from squash (Newberry et al. 2019). The second cluster (B)

gathered the strain P12857_{2ba-B} responsible for VCZ with six cucurbit strains isolated from melon CC457 (Baltrus et al. 2014) and watermelon (13-C2_{2ba-B}, 13-140A_{2ba-B}, 14-410_{2ba-B}, 14-32_{2ba-B}, 14-Gil_{2ba-B}), and finally, cluster C did not contain any VCZ strain but one strain (Zum3584_{2ba-C}) isolated from squash (Newberry et al. 2019). The strain *P. syringae* pv. *syringae* HS191, which was encompassed in clade 2b-a with MLSA, was excluded from one of these clusters. Within clade 2a, the first cluster (D) comprised four VCZ strains (P77_{2a-D}, P73_{2a-D}, P78_{2a-D}, P87_{2a-D}) and two other strains (03-19A_{2a-D}, 200-1_{2a-D}) isolated from melon and squash (Newberry et al. 2019). The second cluster (E) gathered two VCZ strains (P66_{2a-E}, P89_{2a-E}), with strains Bs2121_{2a-E} and Zum3984_{2a-E} isolated from squash (Newberry et al. 2019). The last cluster (F) was composed of two strains (13-139B_{2a-F}, 13-149_{2a-F}) isolated from watermelon (Newberry et al. 2019).

Specific signatures were found in genome sequences with SkIf that distinguish the six clusters containing cucurbit strains within clades 2a and 2b-a. In genomic sequences of strains from clade 2b-a, a total of 108 CDSs with specific signatures were found to differentiate the strains of cluster A from strains of clusters B and C. For cucurbit strains from clade 2a, a total of 578 CDSs with specific signatures of cluster E were found and allowed to distinguish cluster E from clusters D and F. Many specific *k*-mers were identified on CDSs localized in an integrative and conjugative element, but other signatures were located elsewhere in the genome sequences. Among these latter CDSs, three were retained because they were widely distributed within the set of 116 *P. syringae* genome sequences and allowed phylogenetic analyses. These three CDSs corresponded to *Psyr3420*, *Psyr4880*, and *Psyr3208* in strain B728a of *P. syringae*, encoding the nitrogen fixation protein FixH, a hypothetical protein, and the NADH dehydrogenase subunit M, respectively. OrthoMCL analysis revealed proteins specifically present in each cluster comparatively to the others in the same clade.

Fig. 4. Hypersensitive response (HR) of diverse cucurbits after infiltration of vein clearing of zucchini (VCZ) strains. Three VCZ strains (P99, P66, and P108) were infiltrated in leaves of four genotypes of *Cucumis* sp. and one *Citrullus lanatus*. Symptoms were pictured 72 h after inoculation. Pathogenic controls consisted in *Acidovorax citrulli* CFBP 4459 on *Cucumis lanatus* 'Livia', *Pseudomonas syringae* pv. *aptata* CFBP 5467 on melon cultivars, and *P. amygdali* pv. *lachrymans* CFBP6463 on cucumber. The HR control for all tested plants was *P. syringae* pv. *dysoxyl* CFBP 2356. Water was used as a negative control. Within the inoculated area the red line delimits necrosis, and the orange line delimits water-soaked lesion. The white line delimits the expansion of water-soaked symptoms beyond the inoculation point.

Therefore 13, 9, 190, 54, 75, and 384 proteins were specific of clusters A, B, C, D, E, and F, respectively (data not shown).

Proposal of a novel MLSA scheme dedicated to cucurbit pathogens. Seven genes (*gapA*, *gltA*, *gyrB*, *rpoD*, *Psy3420*, *Psy4880*, and *Psy3208*) were selected to highlight all clusters observed with whole genome analyses within clades 2b-a and 2a. The MLSA tree based on the sequences of this novel MLSA scheme including these seven genes was constructed for 23 VCZ strains, 14 strains isolated from cucurbits (Newberry et al. 2019), and 22 diverse strains from phylogroup 2 (Fig. 6A). Phylogenies were constructed for each gene individually and for the concatenated dataset (Supplementary Fig. S3), and their topologies were compared with the SH test. As expected, individual phylogenies were not congruent with the one constructed on the concatenated dataset, indicating that each gene fragment was bringing complementary information. The tree showed that cucurbit strains of clade 2b-a were separated into three distinct branches, and VCZ strains were carried by two of them. The cucurbit strains from clade 2a split also into three separate branches, and VCZ strains were carried by two of them. Overall, composition of each branch was coherent with the composition of clusters identified via whole genome sequence analyses.

T3Es. Strains responsible for VCZ presented different T3E repertoires according to their genetic group (Fig. 7). Using t-BLASTN and sequence alignment, we identified 23 different T3Es in the genome sequences of the 23 VCZ strains. The composition of T3E repertoires was associated with strain clusters identified with whole genome sequence analysis/core-genome MLSA because all strains from a given cluster had homogeneous set of T3Es (Figs. 6B and 7) except two strains from the collection of Newberry, strains

14-Gil_{2ba-B} and 13-139B_{2a-F} which lacked one T3E. The 15 VCZ strains from the cluster A possessed a set of 18 T3Es, and one of them (*avrRpt2*) was specific to that cluster. The strain P12857, which was the only VCZ strain from cluster B, possessed 16 T3Es, of which 14 were common with the cluster A. The four VCZ strains from cluster D possessed 13 T3Es, of which one (*hopA1*) was specific to strains from that cluster. The strains P89_{2a-E} and P66_{2a-E} from cluster E possessed 14 T3Es, 11 being common with cluster D. P108_{2b-S} (singleton) had a smaller T3E repertoire (11 T3Es) with a unique composition but no specific T3E in comparison with the main VCZ clusters. Finally, eight T3Es (*avrE1*, *hopAA1*, *hopAE1*, *hopAZ1*, *hopAG1*, *hopAH1*, *hopBA1*, and *hopI1*) were found in all strains responsible for VCZ.

The different genetic groups of VCZ strains differing by their host ranges are associated with different T3E repertoires (Figs. 6 and 7). Because no pathogenicity data were available for VCZ strains from cluster B, we evaluated the behavior of P12857_{2ba-B} on cucumber, melons, and watermelon with the infiltration method (Supplementary Fig. S4). P12857_{2ba-B} induced spreading water-soaked necrosis in all leaves from these hosts, thus indicating that this strain was pathogenic on these cucurbits. Interestingly, homogeneous T3E repertoires among strains from the same cluster gave homogeneous pathogenicity results (Figs. 6 and 7). The tested strains (P99_{2ba-A}, P79_{2ba-A}) from cluster A (clade 2b-a) were not pathogenic on cucumber, melons, and watermelon and were weakly pathogenic on yellow straightneck genotype. Interestingly, strains from cluster A possessed a unique T3E (*avrRpt2*) and did not have any copy of *hopZ5*, compared with all other VCZ strains from clusters B, D, and E, which were highly pathogenic on these hosts. Strains P66_{2a-E} (cluster E), P12857_{2ba-B} (cluster B), and P108_{2b-S} (singleton) were

Fig. 5. Relationships between genomic sequences of *Pseudomonas syringae* from phylogroup 2 using average nucleotide identity based on BLAST (ANiB) and *k*-mer approaches. Dendrograms were built on distances calculated from ANiB and *k*-mer matrices obtained with Ki-S (Briand et al. 2019). The color blocks represent the cucurbit clusters highlighted with both approaches.

Fig. 6. Phylogeny of vein clearing of zucchini (VCZ) strains, host range, and presence of type III effector (T3E) encoding genes. **A**, Phylogeny based on new multilocus sequence analysis scheme with seven genes (*gapA*, *gltA*, *gyrB*, *rpoD*, *Psyr3420*, *Psyr4880*, and *Psyr3208*). Maximum likelihood tree was based on concatenated partial sequences (4,431 bp). Bootstrap scores (1,000 replicates) were displayed at each node. Black points indicate VCZ strains. **B**, Summarized results obtained from pathogenicity tests and presence of differential genes encoding T3Es recovered from genome sequence analysis. Columns in dark gray report results from Newberry et al. (2018, 2019) that were available for strains Ps711, 13-509A, 13-140A, 13-C2, 14-32, 14-410, 14-Gil, Zum3584, Bs2121, 03-19A, and 200-1. The light gray columns report the results obtained in this study. Colors are as follows: light or dark gray column when a strain is not tested on a given host, red when a strain is pathogenic on a given host, yellow when a strain is weakly pathogenic on a given host, and green when a strain is not pathogenic on a given host. A black box indicates the presence of a T3E. Cucurbit species and subspecies are reported as follows: ME, melons; PU, pumpkin; WM, watermelon; YS, yellow straightneck squash; ZU, zucchini.

pathogenic on watermelon (*Citrullus lanatus* ‘Livia’). It is worth noting that all these strains lacked *hopC1* and *hopH1*, which were present in strains P99_{2ba-A} and P79_{2ba-A} from cluster A, and P73_{2a-D} and P77_{2a-D} from cluster D, strains that were not pathogenic and produced HR-like necrosis on watermelon.

DISCUSSION

Facing the increase of VCZ in zucchini seed-producing areas, we engaged a study to refine results obtained previously on this disease. The present study reveals that VCZ is currently caused by strains belonging to different phylogenetic lineages within the phylogroup 2 of the *P. syringae* species complex. Based on a MLSA using four

housekeeping genes (*gapA*, *gltA*, *gyrB*, and *rpoD*), VCZ strains are grouped mainly in two clades, namely clade 2b-a and clade 2a. A high-resolution analysis based on genome sequences of VCZ strains and cucurbit strains highlights six distinct clusters. VCZ strains belong to four of these six clusters: A and B are associated to clade 2b-a, and D and E are associated to clade 2a. Despite a common disease on zucchini (*Cucurbita pepo* subsp. *pepo*), VCZ strains induce different types of symptoms on other squashes (*Cucurbita pepo* subsp. *texana*, *Cucurbita moschata*, *Cucurbita maxima*) and have a different host range of cucurbit genotypes. Genome sequences were mined to recover genes that matched those clusters. Three largely distributed genes (*Psyr3420*, *Psyr4880*, and *Psyr3208*) were then selected to complete a novel MLSA scheme that can resolve clusters

		Strains	<i>gvfE1</i>	<i>hopAE1</i>	<i>hopAZ1</i>	<i>hopAG1</i>	<i>hopAH1</i>	<i>hopBA1</i>	<i>hopI1</i>	<i>hopAI</i>	<i>hopAA1</i>	<i>hopH1</i>	<i>hopM1</i>	<i>hopZ1</i>	<i>hopBC1</i>	<i>hopAF1</i>	<i>hopR1</i>	<i>hopW1</i>	<i>hopZ5</i>	<i>hopBK1</i>	<i>hopAW1</i>	<i>hopC1</i>	<i>hopAR1</i>	<i>avrRpt2</i>	<i>hopAX1</i>	<i>hopAF1</i>	<i>hopAU1</i>	<i>hopX2</i>	Total T3Es
clade 2b-a	Cluster A	P12832																											18
		P12854																											18
		P12855																											18
		P113																											18
		P118																											18
		P121																											18
		P123																											18
		P127																											18
		P139																											18
		P22																											18
		P5																											18
		P79																											18
		P84																											18
		P12831																											18
		P99																											18
		13-509A																											18
		PS711																											18
clade 2a	Cluster B	P12857																											16
		13-140A																											16
		13-C2																											16
		14-32																											16
		14-410																											16
		14-Gil																											15
		CC457																											16
	Cluster C	ZUM3584																											17
	Cluster E	BS2121																											14
		ZUM3984																											14
		P89																											14
		P66																											14
	Cluster D	P78																											13
		P87																											13
		03-19A																											13
		P73																											13
		P77																											13
Singleton 2b	Cluster F	200-1																											13
		13-139B																											17
Singleton 2b		13-429																											18
		P108																											11

Fig. 7. Set of type III effectors (T3Es) predicted in the genome sequences of vein clearing of zucchini (VCZ) strains and other strains isolated from cucurbits. Color blocks reflect presence of the T3E within a cluster (yellow, cluster A; orange, cluster B; blue, cluster C; green, cluster E; purple, cluster D; gray, cluster F; pink, singleton P108). White block means the absence of T3Es.

of strains pathogenic on cucurbits. The VCZ strains from each cluster share homogeneous T3E repertoires that are associated with pathogenicity features on cucurbit genotypes. Strains from cluster A have a narrow host range and are pathogenic on squashes (*Cucurbita* spp.), whereas VCZ strains from clusters B, D, and E and singleton P108 have a wide host range including squashes, cucumber, and melons. Because they have different host range extents, VCZ strain lineages represent different epidemiological risks in cucurbit-producing areas.

Comparative genomic analyses led to proposing a novel MLSA scheme and differential T3Es that distinguish various lineages of strains that are responsible for diseases on cucurbits. VCZ strains group in four main lineages, whereas a few strains remain isolated in the phylogeny. Other strains responsible for diseases on cucurbits that were previously characterized (Newberry et al. 2019) were included in our genomic analyses. Clearly, all these strains are distant from *P. amygdali* pv. *lachrymans* and cause different diseases. One lineage of VCZ strains (cluster A) presents a narrower host range than other groups, including zucchini, yellow straightneck, butternut, and pumpkin. The presence of a gene encoding AvrRpt2 and the absence of a gene encoding HopZ5 characterize this group of VCZ strains. This lineage includes most strains previously named *P. syringae* pv. *peponis* (Manceau et al. 2011) and is included in clade 2b-a, which is described as being the result of recombination events between *P. syringae* and *P. cerasi* (Newberry et al. 2019). The three other groups of VCZ strains (clusters B, D, and E) are distributed in clades 2a and 2b-a. Clade 2a corresponds to *P. cerasi* (Newberry et al. 2019). We show here that VCZ strains of these three groups are pathogenic not only on zucchini but also on yellow straightneck, butternut, pumpkins, cucumber, melons, and in some cases watermelon. These groups include also strains that were independently shown to induce angular leaf spot and bacterial leaf spot of watermelon, cantaloupe, and squash by Newberry et al. (2016, 2018, 2019). The presence of the gene encoding HopZ5 and the absence of the gene encoding AvrRpt2 in their genome sequences characterize these lineages. Taking into account that the diseases induced in zucchini by the four groups of strains and the singleton (P108_{2b-S} in clade 2b corresponding to *P. syringae* *sensu stricto*) are similar, whereas the host range of cluster A is different from the one of clusters B, D, and E; that these strains are distributed over different species; and that we did not test on our cucurbit genotypes the pathotype strain of *P. syringae* pv. *syringae*, known to have a large host range, we cannot conclude that the four lineages truly form distinct pathovars. It should be noted that the singleton P108_{2b-S} is phylogenetically close to *P. syringae* pv. *aptata*, which includes strains isolated from melons (Berge et al. 2014). Strain CFBP 5427 from this pathovar induces symptoms on squash, cucumber, and melons that are similar to those caused by the P108_{2b-S} strain. The only differences are the inability of the *P. syringae* pv. *aptata* CFBP 5427 strain to induce stunting of zucchini plantlets with the seed soaking test and the susceptibility of watermelon to P108_{2b-S} but not to CFBP 5427.

Specific T3Es could be used as genetic markers of differential host ranges of VCZ strains. In this study, we identified that the presence of *hopZ5* and absence of *avrRpt2* are common characteristics of strains from the three clusters that share a wide host range and are pathogenic on melons and cucumber. In contrast, cluster A strains have *avrRpt2* and lack *hopZ5* and are not pathogenic on these groups of genotypes. Further experimental work is necessary to decipher the role of these T3Es in host range extents. Interestingly, it was also shown that strains from clusters C and F harbor *hopZ5* and are pathogenic on squash, melon, and watermelon (Newberry et al. 2019). *HopZ5* was previously proposed to promote virulence among strains pathogenic on cucurbits and having different T3E repertoires (Newberry et al. 2019). Here we suggest that *hopZ5* could also be a good predictor of a wide host range on cucurbits. Cluster A strains (Ps711_{2ba-A} and

13-509A_{2ba-A}) harboring *avrRpt2* and missing *hopZ5* have been previously reported to be weakly pathogenic on squash, melon, and watermelon (Newberry et al. 2018, 2019). Their pathogenicity was evaluated on a yellow straightneck genotype, but strains were not tested on cucumber or on any other genotypes of squashes, including zucchini. Therefore, this weak pathogenicity on yellow straightneck for cluster A strains is consistent with the dried brown lesions we observed for strains P99_{2ba-A} and P79_{2ba-A} on yellow straightneck. Indeed, the dried lesions seemed less severe than the water-soaked lesions caused by strains of clusters B, D, and E and the singleton. The gene *avrRpt2* is commonly found in *P. syringae* pv. *tomato* strains (Almeida et al. 2009) and has been shown to promote pathogenicity by stimulating key regulators in auxin signaling pathways (Cui et al. 2013). This effector leads to HR-like necrosis in *A. thaliana* through the cognate RPS2 resistance gene (Pike et al. 2005). Apart from the main genetic clusters including VCZ strains, we also characterized a singleton (P108_{2b-S}), which is pathogenic on all tested cucurbits and induces VCZ. P108_{2b-S} is close to *P. syringae* pv. *aptata* strains in the phylogenetic analysis. This singleton has fewer T3Es (11 T3Es) than other VCZ strains. Eight T3Es (*avrE1*, *hopAA1*, *hopAE1*, *hopAZ1*, *hopAG1*, *hopAH1*, *hopBA1*, and *hopI1*) are common to all the VCZ strains and might be associated to pathogenicity on zucchini. It is also interesting to note that Newberry et al. (2016, 2019) characterized singletons from clade 2b and 2d isolated from diseased cucurbits. Previous studies also identified a potential large host range for strains from clade 2d (Monteil et al. 2016; Morris et al. 2019).

Not all VCZ strains were pathogenic on watermelon cultivar Livia because tested strains from clusters A (P99_{2ba-A} and P79_{2ba-A}) and D (P73_{2a-D} and P77_{2a-D}) did not induce any disease with the spray inoculation and induced HR-like necrosis when infiltrated in leaves. These results were also obtained with cultivar Troubadour (data not shown). Based on genome analysis, strains Ps711_{2ba-A} and 13-509A_{2ba-A} belong to cluster A, and strains 200-1_{2a-D} and 03-19A_{2a-D} belong to cluster D. Interestingly, these strains were previously tested with a similar spray inoculation and were shown to be weakly pathogenic or not pathogenic on watermelon cultivars Troubadour and Wrigley (Fig. 6B) (Newberry et al. 2016, 2017, 2019). These discrepancies on watermelon could be strain or test dependent. However, some effectors are associated to strains from clusters A and D that are weakly pathogenic or not pathogenic on watermelon because all possess complete genes of *hopH1* and *hopC1*, in contrast to other aggressive strains from clusters B, C, E, F, and singleton. This result is consistent with those previously reported (Newberry et al. 2019).

The use of several pathogenicity tests permitted a precise phenotypic characterization of strains responsible for VCZ. On zucchini, the cotyledon rubbing test successfully reproduces the typical symptoms (necroses on leaves and cotyledons, vein clearing, and stunting) observed in seedling-producing areas and caused by VCZ strains. The cotyledon rubbing test is quick and easy to perform, but it induces microinjuries on plantlets by trichome breaks and thus creates openings. For this reason, the cotyledon rubbing test is invasive and can produce biases to study bacterial strains' pathogenicity (Burdman et al. 2005; Klement et al. 1990). The seed soaking tests were appropriate to study VCZ strains on their host of origin and permitted to properly characterize strain aggressiveness. Seed soaking tests are consistent with VCZ strains being described as seedborne bacteria (Manceau et al. 2011). Seed soaking tests are commonly used to characterize aggressiveness of seedborne bacteria such as *A. avenae* subsp. *citulli* on cucurbits (Burdman et al. 2005). Then, the spray inoculation method was used to simulate natural infection of leaves by pathogen and allowed bacterial entry through natural openings (Klement et al. 1990). This test was particularly relevant to finely characterize symptoms and host range. Finally, the HR test is an easy, fast, and efficient method to define host range of VCZ strains on different cucurbit genotypes. Host incompatibility produces an HR-like reaction on leaves 48 h

postinoculation, whereas host compatibility produces spreading water-soaked necrosis.

The strains responsible for VCZ were isolated from plantlets issued from zucchini seed lots produced in distant regions over time. Only a few studies have dealt with *P. syringae* strains transmitted by cucurbit seeds (Dutta et al. 2014; Meng et al. 2016; Shila et al. 2013). Strains from *P. syringae* pv. *aptata*, *P. syringae* pv. *syringae*, and *P. amygdali* pv. *lachrymans* were reported to be seed disseminated, and efforts to develop seed detection tests contribute to improved pathogen control and disease management. We identified specific markers (*Psyr3420*, *Psyr4880*, and *Psyr3208*) that can be used to design specific VCZ strain primers. A specific detection tool is indeed needed to conduct epidemiological studies of VCZ in worldwide zucchini seed production areas. Typing *P. syringae* isolates from diseased cucurbits with the new MLSA scheme would give insight into worldwide dissemination of strains and origin of epidemics. The gain of knowledge on VCZ strains and the survey of epidemics are essential steps to implement efficient management of the various VCZ lineages, which do not represent the same risk as a consequence of their different host range.

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