Characterization, Phylogeny, and Genome Analyses of Nonpathogenic *Xanthomonas campestris* Strains Isolated from Brassica Seeds

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ABSTRACT

Xanthomonads were detected by using the Xan-D(CCF) medium from the brassica seeds, and their pathogenicity was determined by plant inoculation tests. It was found that some seed lots were infested with *Xanthomonas campestris* pv. *campestris*, some with *X. campestris* pv. *raphani*, and some with nonpathogenic xanthomonads. The nonpathogenic xanthomonad strains were identified as *X. campestris*, and the multilocus sequence analysis showed that the nonpathogenic *X. campestris* strains were grouped together with pathogenic *X. campestris*, but not with nonpathogenic strains of *X. arboricola*. In addition, all isolated *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* strains were positive in the *hrpF*-PCR, but the nonpathogenic strains were negative. It was further found that nonpathogenic *X. campestris* strain nE1 does not contain the entire pathogenicity island (*hrp* gene cluster; type III secretion system) and all type III effector protein genes based on the whole genome sequence analyses. The

nonpathogenic *X. campestris* strain nE1 could acquire the entire pathogenicity island from the endemic *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* strains by conjugation, but type III effector genes were not cotransferred. The studies showed that the nonpathogenic *X. campestris* strains indeed exist on the brassica seeds, but it could be differentiated by the PCR assays on the *hrp* and type III effector genes. Nevertheless, the nonpathogenic *X. campestris* strains cannot be ignored because they may be potential gene resources to increase genetic diversity in the endemic pathogenic *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* strains.

Keywords: bacteriology, disease control and pest management, pathogenicity island, plant seed detection, type III secretion system, type III effector, *Xanthomonas campestris*

The plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* is a seedborne bacterium that causes black rot of crucifers. Contaminated plant seeds are an important primary source of inoculum for the bacteria (Roberts et al. 1999), and the use of pathogen-free seeds is critical to control the bacterial disease. Seed assays to detect *X. campestris* pv. *campestris* are therefore routinely performed in many laboratories. Some semiselective media are recommended in Working Sheets in the ISTA Handbook on Seed Health Testing (Schaad and Franken 1996), but several yellow non-xanthomonads associated with plant tissues could still grow on the media and interfere with the detection and identification. An xanthomonad differential medium (Xan-D medium) was previously developed to differentiate xanthomonads from yellow non-xanthomonads and is successfully used to isolate the viable xanthomonads from naturally diseased cabbages and infested cabbage seeds (Lee et al. 2009).

The pathogenic xanthomonads of cruciferous vegetables include not only *X. campestris* pv. *campestris* (black rot pathogen) but also *X. campestris* pv. *raphani* (leaf spot pathogen) (Fargier and Manceau 2007). In addition, there is growing evidence that many nonpathogenic (or commensal) xanthomonads could exist on plant surfaces or seeds and cause no disease symptoms on the host from which they were isolated (Vauterin et al. 1996). The nonpathogenic xanthomonads have been reported to occur on rice seeds (Ming et al. 1991). Because the detection of *X. campestris* pv. *campestris* or *X. campestris* pv. *raphani* on cruciferous vegetable seeds, such as cabbage and cauliflower, is an important plant quarantine measure

for the management of diseases, the existence of nonpathogenic xanthomonads on the seed lots may complicate plant quarantine and disease treatment.

The developed semiselective media for xanthomonads could not differentiate the pathogenic and nonpathogenic, and the pathogenicity of xanthomonads should be further determined by time-consuming plant inoculation tests. In practical diagnosis, it is important to identify the nonpathogenic in a rapid manner. The *hrp* (hypersensitive response and pathogenicity) genes, coding for the type III secretion system (T3SS), have been used to identify pathogenic and nonpathogenic *X. campestris* pv. *vesicatoria* (Leite et al. 1994). Among *hrp* genes, *hrpF* was reported to contribute to pathogenicity and/or virulence of pathogenic xanthomonads and was used to develop a PCR-based detection method of *X. campestris* pv. *campestris* (Berg et al. 2005; Park et al. 2004). It was also found that some *X. campestris* pv. *campestris* strains isolated from cruciferous horseradish failed to amplify any DNA products using *hrpF* primers and also did not develop infection on plant hosts (Berg et al. 2005).

In this paper, we determined the identity of *X. campestris* strains isolated from the brassica seeds and report that nonpathogenic *X. campestris* strains that are free of T3SS indeed exist on the seeds. The multilocus sequence analysis was further used to determine the phylogenetic relationship between the isolated nonpathogenic *X. campestris* strains and the previously identified nonpathogenic *X. campestris* and *X. arboricola* strains (Fargier et al. 2011; Garita-Cambronero et al. 2017), which provides useful information about the emergence and distinction of pathogenic strains of *X. campestris*. The occurrence of nonpathogenic *X. campestris* on the cruciferous seeds may challenge the current seed detection methods of *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*, and also raise the question of what genes the nonpathogenic *X. campestris* lacks regarding the pathogenicity, and whether the gene transfer might occur between the nonpathogenic and pathogenic *X. campestris*. Whole genome analysis was used to compare the nonpathogenic with pathogenic

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X. campestris, especially focusing on the genes of pathogenicity, and the possibility of gene transfer between them was also determined. This study further provides some genetic markers to differentiate nonpathogenic and pathogenic *X. campestris* after isolation from brassica seeds.

MATERIALS AND METHODS

Isolation and characterization of xanthomonads isolated from brassica seeds. The brassica seeds were assayed for xanthomonads using a seed-wash dilution plating assay. Briefly, 3 g of seeds per seed lot was incubated in 10 ml of washing buffer (0.85% NaCl; 0.02% Tween 80) in a 120-ml flask. The flasks were placed on a rotary shaker (125 rpm) at 28°C for 5 h. The seed wash was serially diluted tenfold. Then 100 µl of each dilution was plated onto the plates of Xan-D(CCF) medium (Lee et al. 2009) and spread over the surface with a sterile bent glass rod. An additional 1 ml of seed wash was centrifuged at 13,000 × g for 1 min and a 900-µl supernatant was removed. The resulting pellet was resuspended in 100 µl of the remaining water buffer and spread onto the plates of Xan-D(CCF) medium. Plates were incubated at 28°C and observed for the presence of typical colonies (wet-shining yellow-green with milky and clear zones) of xanthomonad for 3 to 5 days.

Some seed lots were contaminated with *Stenotrophomonas* spp., such as *S. maltophilia*, whose colony is also yellow-green, but nonmucoid and raised with undulate margin on the Xan-D(CCF) medium (Lee et al. 2009). The colonies were then subcultured to the YDC medium (1% yeast extract, 2% dextrose, 2% CaCO₃, and 1.5% Bacto-agar) (Wilson et al. 1967) and tested by *estA*-PCR using the Xc-lip-F2/Xc-lip-R2 primer set (Lee et al. 2009). Xanthomonads are yellow and mucoid and positive in the *estA*-PCR, but *Stenotrophomonas* spp. are pale or light yellow and flat on the YDC medium and negative in the PCR.

Pathogenicity test. The pathogenicity of the isolated xanthomonads was tested by inoculating cabbage seedlings (*Brassica oleracea* L. var. *capitata*) by cutting leaf margins with a pair of sterilized scissors that had been dipped in a suspension of bacteria containing 10⁶ to 10⁷ CFU/ml. The seedlings were also inoculated by dusting Carborundum on the leaves and then rubbing leaves with the bacterial suspension (10⁶ to 10⁷ CFU/ml). The inoculated plants were covered with a plastic bag in a growth chamber at 26°C under conditions of 16 h of light and 8 h of darkness. After 24 h, the plastic bag was removed and plants were maintained in the growth chamber and symptoms were evaluated after 7 to 14 days.

Phylogenetic analysis of isolated nonpathogenic xanthomonads. The isolated xanthomonads that did not cause any symptoms on cabbage were considered nonpathogenic xanthomonads. To further determine their identity and phylogenetic relationship with pathogenic *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*, the colonies were subjected to PCR assays to obtain their partial 16S rRNA and *gyrB* gene fragments using 16S-Fm (5'-AGTTTGATCCTGGCTCAGAGTGAA-3')/16S-R4 (5'-GCACCTTCCGATACGGCTACCTTG-3') and Xgyr-PCR2F/Xgyr-rsp1 (Parkinson et al. 2007) as primer sets, which yield about 1,520- and 730-bp DNA fragments, respectively. The DNA fragments were ligated into pGEM-T Easy vectors (Promega Corp., Madison, WI), and double-stranded DNA sequencing was performed with standard universal T7 and SP6 primers. The 16S rRNA sequences were analyzed using sequence analysis tools and the database of the National Center for Biotechnology Information (NCBI). The nucleotide sequence of *gyrB* was trimmed in silico at positions corresponding to the 5'-end ATCACCGGCG and 3'-end GAGCAGTTGTG to generate 530-bp long sequences (Parkinson et al. 2007), and multiple sequence alignments were performed using ClustalW (Thompson et al. 1994) with the trimmed sequences of other xanthomonads from GenBank. The phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei 1987) by using a MEGA4 program (Tamura et al. 2007).

The multilocus sequence analysis (MLSA) of four housekeeping genes, *dnaK* (heat shock protein 70, molecular chaperone DnaK), *fyuA* (TonB-dependent receptor), *gyrB* (DNA gyrase subunit B), and *rpoD* (RNA polymerase sigma-70 factor), was further used to analyze the phylogenetic relationship between the isolated nonpathogenic *X. campestris* strains, nE1 and nT1, and other nonpathogenic and pathogenic *X. campestris* strains from brassica crops (Fargier et al. 2011). The MLSA also included the nonpathogenic (T3SS-negative) and pathogenic *X. arboricola* strains isolated from *Prunus* (Garita-Cambronero et al. 2017) in order to understand the association between nonpathogenic xanthomonads and their plant hosts. The partial sequences of four genes of *X. arboricola* strains are obtained from GenBank based on their accession numbers indicated in Garita-Cambronero et al. (2017). The gene sequences of *X. campestris* pv. *campestris* strain ATCC 33913 (NC_003902.1), *X. campestris* pv. *raphani* 756C (NC_017271.1), *X. campestris* pv. *incanae* CFBP 2527R (NZ_CM002636.1), and *X. campestris* pv. *barbareae* CFBP 5825 (nonpathogenic; NZ_CM002638.1) were obtained from their genome sequences in the NCBI database. The sequences of the isolated nonpathogenic *X. campestris* strain nE1 are from the assembled contigs after whole genome sequencing as described below and those of nonpathogenic *X. campestris* strain nT1 were obtained by sequencing the PCR products as described in Young et al. (2008). Each sequence of four housekeeping genes was trimmed to the following size: *dnaK*, 842 positions; *fyuA*, 601 positions; *gyrB*, 631 positions; and *rpoD*, 759 positions, and sequences were concatenated to give a total length of 2,833 positions according to Garita-Cambronero et al. (2017). Multiple alignments of the concatenated nucleotide sequences and the construction of phylogenetic trees were performed as described above.

Draft genome analysis of a nonpathogenic *Xanthomonas* sp. strain. The genome of nonpathogenic *X. campestris* strain nE1 was sequenced with 2 × 300-bp paired-end sequencing of a shotgun library on an Illumina MiSeq system. After quality trimming of the raw sequencing reads and assembly of the trimmed sequencing reads into contigs, the assembled contigs of strain nE1 were compared with the genome sequences of *X. campestris* pv. *campestris* ATCC 33913 (accession number NC_003902) and *X. campestris* pv. *raphani* 756C (accession number CP002789) using DNASTAR Lasergene 12.1 (DNASTAR, Inc., Madison, WI) to determine the existence of type-III secretion system encoded by the *hrp* gene cluster (pathogenicity island) and type III effector genes. Because most of type III effector genes are distributed in different genomic regions, the nucleotide sequence of each type III effector gene currently identified in *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* (Ryan et al. 2011) was used to search for the homolog in the assembled contigs. In addition, two transcriptional activators of *hrp* genes, *hrpG* and *hrpX*, located outside the pathogenicity island, were also searched.

Conjugation between pathogenic and nonpathogenic *X. campestris* strains. To determine whether the pathogenicity island can be transferred by conjugation from the endemic Xcc1-1 and Xcr-R1 strains to the nonpathogenic *X. campestris* strain nE1. A selection marker, tetracycline resistance gene (Tc^R), was first inserted into the *hrpF* of pathogenicity island in Xcc1-1 and Xcr-R1 strains by transposon mutagenesis and marker exchange. A partial *hrpF* DNA fragment (1,906 bp) was amplified from the Xcc1-1 strain by a PCR assay using hrpF-2-F/hrpF-2-R as primers and ligated into pGEM-T Easy vectors (Promega Corp.). The pGEM-Teasy-*hrpF* was subjected to mutagenesis with EZ::Tn5™ <TET-1> transposon insertion kit (EPICENTRE Biotechnologies, Madison, WI) according to the directions of the manufacturer. A plasmid with a transposon inside *hrpF* was selected and designated as pGEM-Teasy-*hrpF*::Tn5<TET-1>. The plasmid was further introduced into endemic Xcc1-1 and Xcr-R1 strains, which were isolated from diseased cabbage and radish more than 15 years ago,

by electroporation (Yang et al. 1991). Double recombinant clones, called Xcc1-1 (*hrpF*::Tn5<TET-1>) and Xcr-R1 (*hrpF*::Tn5<TET-1>), were selected on the basis of resistance to tetracycline and further verified by the *hrpF*-2 PCR assay.

Strains Xcc1-1 (*hrpF*::Tn5<TET-1>) and Xcr-R1 (*hrpF*::Tn5<TET-1>), which contain the tetracycline resistance gene in the *hrpF* of pathogenicity island, were used as donor bacteria, and the nE1 rifampicin-resistant (Rif^R) strain as a recipient bacterium in the conjugation test. Both donor and recipient bacteria were grown overnight at 28°C on Luria Bertani (LB) agar medium (Miller 1972) containing tetracycline (12.5 µg/ml) or rifampicin (75 µg/ml). Bacteria were collected from solid media and cultured again in LB broth without antibiotics for 6 h. Equal volume (750 µl) of donor and recipient bacteria were mixed in a tube and centrifuged briefly (approximately 30 s), and the supernatant was discarded. The bacterial pellets were suspended in 50 µl of sterile tap water by pipetting, plated on the nutrient yeast extract-glycerol agar (NYGA; 0.5% Bacto-peptone, 0.3% yeast extract, 2% glycerol, and 1.2% Bacto-agar) (Daniels et al. 1984), and incubated at 28°C for 16 to 24 h. The bacteria were scraped from the NYGA plates, suspended in 50 to 100 µl of sterile tap water and spread onto the LB agar medium with tetracycline and rifampicin at 28°C for 3 to 14 days. The putative transconjugant colonies growing on the medium were selected and determined by the PCR assay using (i) the Tc-F/Tc-R primer set for the tetracycline resistance gene of Tn5<TET-1> inside the *hrpF*, which could be transferred from the donor bacteria, and (ii) the *thuA*-F/*thuA*-R and *akr*-F/*akr*-R primer sets (Table 1) to confirm the identity of strain nE1, which are designed based on the *thuA* domain-containing protein (TR80_004955) and aldo/keto reductase family oxidoreductase (TR80_015515) genes only existing in strain nE1 but not in the Xcc1-1 and Xcr-R1 strains. The PCR-positive strain nE1 transconjugants were further tested by PCR to determine the existence of pathogenicity island transferred from the donor bacteria.

PCR assays. The PCR primers used in this study are listed in Table 1. The colonies of xanthomonads on the LB agar medium were suspended in 50 µl of sterile distilled water. The bacterial suspension was incubated for 10 min at 100°C for cell lysis, and was maintained at 4°C before use. The PCR amplification was performed with a Thermal cycler 2720 (Applied Biosystems, Foster City, CA) in a 30-µl reaction mixture containing 1 µl of cell lysate, 3 µl of primer pair (0.35 to 0.5 µM of each primer), 15 µl of GoTaq green master mix (Promega Corp.), and 11 µl of sterile distilled water by using the following program: 1 cycle of denaturation for 1 min at 94°C; 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Reactions were stored at 15°C until they were used for analysis. Amplified DNA was detected by electrophoresis in 1.0% agarose (agarose I, Amresco Inc., Solon, OH) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

Nucleotide sequence accession number. The 16S rRNA partial sequences of nonpathogenic *X. campestris* strains nE1 and

nT1 have been assigned GenBank accession numbers KT156666 and KT156667, respectively. The *gyrB* sequences of those isolates were assigned KT191043 and KT191044, the *hrpG* were assigned MK858167 and MK858168, and the *hrpX* were MK878420 and MK878419. The partial sequences of the four housekeeping genes used in the MLSA are MN882614 and MN882615 for *dnaK*; MN882616 and MN882617 for *fyuA*; MN882618 and MN882619 for *gyrB*; and MN882620 and MN882621 for *rpoD*. This whole genome shotgun project of nXan-E1 has been deposited at DDBJ/EMBL/GenBank under accession number JXYR000000000. The version described in this paper is version JXYR02000000.

RESULTS

Isolation, characterization, and pathogenicity test of xanthomonads isolated from brassica seeds. The Xan-D(CCF) medium was used to detect the xanthomonads from 50 seed samples (30 cabbage, 15 cauliflower, and 5 broccoli seed samples) in 2013 to 2016. The xanthomonad-like bacterial colonies, which were yellow-green, wet-shining, convex and surrounded by milky and clear zones on the medium, were isolated from nine seed samples after incubation for 3 to 4 days at 28°C. Two characteristic colonies isolated from separate seed samples were subcultured on the YDC medium and also tested by a PCR assay using the *estA* (Xc-lip-F2/Xc-lip-R2) primer set. The isolates that were yellow, mucoid and convex on the YDC medium and positive in the *estA*-PCR were considered xanthomonads (Fig. 1A) (Lee et al. 2009; Wilson et al. 1967). Some seed samples were contaminated with *Stenotrophomonas* spp., such as *S. maltophilia*, whose colony is also yellow-

Fig. 1. Polymerase chain reaction analysis of isolated xanthomonads from brassica seeds using the primer sets designed from **A**, the esterase gene, *estA* (Xc-lip-F2/Xc-lip-R2; 777 bp), and **B**, the pathogenicity island gene, *hrpF* (hrpF-1-F/hrpF-1-R; 424-bp). Lanes 1 to 3: *X. campestris* pv. *campestris* (Xcc) strains Xcc1-1, Xcc-G, and Xcc-T1; lanes 4 to 6: *X. campestris* pv. *raphani* (Xcr) strains Xcr-X2B1, Xcr-X3A2, and Xcr-TGA; and lanes 7 to 9: non-pathogenic xanthomonad (nXan) strains nE1, nT1, and nA1. M, molecular size marker (1-kb Plus DNA ladder, Invitrogen Life Technologies, Carlsbad, CA).

TABLE 1. Primers used for PCR amplifications

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)
<i>avrBs2</i>	TGCCGGTGTGATGCACGA	TCGGTCAGCAGGCTTTC	850
<i>hrcR</i>	GAAAGGCCATCGCCATCA	TCACCTCCTACCAAGAT	619
<i>hrpF</i> -1	TGATCGCTATTCGACTATCTGCC	TTCTTGATGCCGGTGATCGAGTTGTTC	424
<i>hrpF</i> -2	ATGTCGCTCAACACGCTTTCTACGGGAG	TTCTTGATGCCGGTGATCGAGTTGTTC	1,906
<i>hrpG</i>	TCGCAGGTCAACGCCAGCCT	GCTCCATGGTGCGGTCGGTG	544
<i>Tc</i>	AGCATCGCCAGTCACTATGGCG	GGCCTGCAACGCGGGCATCC	849
<i>xopAT</i>	CGAACAGTGTAATGTGGGG	CGATACTCTCTTCTGGGTCTG	325
<i>xopP</i>	GGCGCCGGATACACACAAG	AGCTGCAAACGCTCGGTGA	954
<i>thuA</i> ^a	CGGATGCTGATCTTCAGCAAGACGGC	GCCGCATAGCGCAGCC	629
<i>akr</i> ^a	CATCCGCCTCTACCTATGCCCTCG	CGCGGACTGCAGTGGCGAG	659

^a Nonpathogenic *Xanthomonas campestris* strain nE1 specific genes: *thuA* (TR80_004955), *ThuA* domain-containing protein, and *akr* (TR80_015515), aldo/keto reductase family oxidoreductase.

green, but nonmucoid on the Xan-D(CCF) medium (Lee et al. 2009). The *S. maltophilia* isolates are pale or light yellow and flat on the YDC medium and negative in the *estA*-PCR.

The pathogenicity of isolated xanthomonads was tested by the inoculation test. Some isolates that caused black rot symptoms on cabbage leaves were considered as *X. campestris* pv. *campestris*, and the isolates causing leaf spot symptoms were *X. campestris* pv. *raphani*. The other isolates did not cause any visible symptoms and were presumptively considered nonpathogenic xanthomonads. The *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* strains produced a 424-bp DNA fragment in the *hrpF*-PCR reaction using the *hrpF* primer set (*hrpF*-1-F/*hrpF*-1-R). All nonpathogenic xanthomonads tested did not amplify any DNA fragment in the *hrpF*-PCR (Fig. 1B). The two cabbage and two cauliflower seed samples were infested with *X. campestris* pv. *campestris*, two cabbage seed samples with *X. campestris* pv. *raphani*, and two cabbage and one broccoli seed samples with nonpathogenic xanthomonads. Each infested seed sample contained only one strain of *X. campestris* pv. *campestris*, *X. campestris* pv. *raphani*, and nonpathogenic xanthomonads. The bacterial strains are listed in Table 2.

Further identification and phylogenetic analysis of nonpathogenic xanthomonads. In addition to the characteristic growth features on the Xan-D and YDC media, and the positive reaction in *estA*-PCR, the nucleotide sequence of 16S rRNA was further used to confirm the identity of isolated nonpathogenic xanthomonads. Almost complete nucleotide sequences of 16S rRNA of nonpathogenic xanthomonad strains, nE1 and nT1, were determined and compared with the available 16S rRNA sequences in GenBank. The sequences are highly identical (99 to 100%) to those of *X. campestris* pv. *campestris* ATCC 33913 (accession number NR_074936) and *X. campestris* pv. *raphani* 756C (CP002789), *X. axonopodis* pv. *citri* 306 (NR_074937), and *X. oryzae* pv. *oryzae* KACC10331 (NR_074938). Accordingly, the isolated nonpathogenic xanthomonad strains should be assigned to the genus *Xanthomonas*.

The partial *gyrB* nucleotide sequence of two nonpathogenic xanthomonad strains was determined and used to construct the phylogenetic tree (Fig. 2). The nonpathogenic xanthomonad strains, nE1 and nT1, aligned closely with *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*. In addition, the complete *gyrB* sequences

of nE1 and nT1 strains were also determined and shared more than 98% identity with those of *X. campestris* pv. *campestris* ATCC 33913 (AE008922) and *X. campestris* pv. *raphani* 756C (CP002789), and only 91 to 92% with other *Xanthomonas* spp. Therefore, the isolated nonpathogenic xanthomonads should belong to the species *X. campestris*, and are called nonpathogenic *X. campestris* hereafter.

The MLSA analysis showed that *X. campestris* strains from brassica crops formed a monophyletic group, and clearly separated from another group formed by the *X. arboricola* strains isolated from *Prunus* hosts. Each group was further separated into two subgroups, one with T3SS and the other without the system (Fig. 3). Two isolated nonpathogenic *X. campestris* strains, nE1 and nT1, which are free of T3SS as described below, were grouped together with the T3SS-positive strains of *X. campestris*, but not with T3SS-negative strains of *X. arboricola*. In *X. campestris* group, *X. campestris* pv. *barbareae* CFBP 5825 is considered nonpathogenic, but it contains T3SS (Roux et al. 2015) and is clustered with the pathogenic and T3SS-positive strains of *X. campestris*,

Fig. 2. Unrooted phylogenetic tree derived from neighbor-joining analysis of the partial *gyrB* nucleotide sequences of the nonpathogenic xanthomonad strains, nE1 and nT1, isolated from brassica seeds, *Xanthomonas* spp., and two closely related bacteria, *Xylella fastidiosa* and *Stenotrophomonas maltophilia*. Bootstrapping resampling statistics were applied to the tree (1,000 data sets), and bootstrap values are shown on each horizontal branch of the tree. The superscript T indicates that the strain is the type strain. GenBank accession numbers are given in parentheses after the bacterial names.

TABLE 2. Bacterial strains tested

Seed lots	Plants	Strains ^a	Pathogenicity	PCR ^b			
				<i>estA</i>	<i>hrpG</i>	<i>hrpF</i>	<i>avrBs2</i>
CA05	Cabbage seed	Xcc-G	+	+	+	+	+
CA05	Cabbage seed	Xcc-T1	+	+	+	+	+
CA18	Cabbage seed	Xcc-X3LB	+	+	+	+	+
CA18	Cabbage seed	Xcc-TG1	+	+	+	+	+
CL16	Cauliflower seed	Xcc-05-1	+	+	+	+	+
CL16	Cauliflower seed	Xcc-06	+	+	+	+	+
CL34	Cauliflower seed	Xcc-P1	+	+	+	+	+
CL34	Cauliflower seed	Xcc-P5-1	+	+	+	+	+
CA23	Cabbage seed	Xcr-X2B1	+	+	+	+	—
CA23	Cabbage seed	Xcr-X3A2	+	+	+	+	—
CA46	Cabbage seed	Xcr-TGA	+	+	+	+	—
CA46	Cabbage seed	Xcr-TH1B	+	+	+	+	—
CA26	Cabbage seed	nE1 ^c	—	+	+	—	—
CA26	Cabbage seed	nT1	—	+	+	—	—
CA43	Cabbage seed	nS1	—	+	+	—	—
CA43	Cabbage seed	nN1	—	+	+	—	—
BR03	Broccoli seed	nA1	—	+	+	—	—
BR03	Broccoli seed	nA2	—	+	+	—	—
—	Diseased cabbage	Xcc1-1 ^d	+	+	+	+	+
—	Diseased radish	Xcr-R1 ^d	+	+	+	+	—

^a Xcc and Xcr indicate *Xanthomonas campestris* pv. *campestris* and *X. campestris* pv. *raphani*, respectively.

^b The PCR assays used to differentiate *X. campestris* pv. *campestris*, *X. campestris* pv. *raphani*, and nonpathogenic *X. campestris* as shown in Figures 1 and 6.

^c The n indicates nonpathogenic for the isolated *X. campestris* strains.

^d *X. campestris* pv. *campestris* Xcc1-1 and *X. campestris* pv. *raphani* Xcr-R1 were isolated from diseased cabbage and radish in Taiwan 15 years ago and are considered endemic strains.

including *X. campestris* pv. *campestris*, *X. campestris* pv. *raphani*, and *X. campestris* pv. *incanae*.

Draft genome analysis of nonpathogenic *X. campestris* strain nE1. Because all isolated nonpathogenic *X. campestris* strains did not amplify any DNA fragment in the *hrpF*-PCR, the lack of pathogenicity might not be attributed only to the absence of *hrpF*. Whole genome sequence of strain nE1 was determined and analyzed

to further understand whether the nonpathogenic *X. campestris* strain also lack other pathogenicity-related genes. The genome sequencing data of strain nE1 are made up of 4.3 million reads of 208-bp average length after quality trim, generating 891-Mb raw data with a G+C content of 64%. The raw coverage depth is therefore 178× over average xanthomonad genome (5 Mb). The reads were assembled into 15 contigs (N50 = 242.8 kb) with the sum of 5.0 Mb. Although the contigs do not overlap each other to form a circular genome of the bacterium, the total length of the contigs is almost close to the genome size of *X. campestris* pv. *raphani* 756c (4.941 Mb) and *X. campestris* pv. *campestris* ATCC 33913 (5.076 Mb).

The contigs of strain nE1 were mapped to the genome sequences of *X. campestris* pv. *campestris* ATCC 33913 (NC_003902) and *X. campestris* pv. *raphani* 756c (CP002789). It was found that all contigs of strain nE1 were not aligned to a DNA fragment containing *hrp* gene cluster (pathogenicity island; T3SS) of either *X. campestris* pv. *campestris* ATCC 33913 or *X. campestris* pv. *raphani* 756c. A 44.5-kb DNA fragment of *X. campestris* pv. *campestris* ATCC 33913 with pathogenicity island is absent in the strain nE1 (Fig. 4). The DNA fragment of *X. campestris* pv. *campestris* ATCC 33913 starts from XCC1208 (glyoxalase family protein) to XCC1247 (*xopP*), includes a 23.1-kb pathogenicity island, which is from XCC1217 (*hrpF*) to XCC1241 (*hpa2*), and also contains one putative effector protein gene, XCC1247 (*xopP*).

A 59.2-kb DNA fragment of *X. campestris* pv. *raphani* 756c strain containing the pathogenicity island does not exist in the strain nE1 (Fig. 4). The DNA fragment begins from XCR_1448 to XCR_1505 (periplasmic beta-glucosidase), covering a 37.8-kb pathogenicity

Fig. 3. Phylogenetic tree derived from neighbor-joining analysis of the concatenated sequences of the genes *dnaK*, *fyuA*, *gyrB*, and *rpoD* of non-pathogenic *Xanthomonas campestris* strains, nE1 and nT1, isolated in this study and other strains of *X. campestris* isolated from brassica crops and those of *X. arboricola* from prunus. The presence or absence of type III secretion system (T3SS), T3SS (+) or (-), of *X. campestris* pathovars and strains are based on Merda et al. (2017), Roux et al. (2015), and this study, and those of *X. arboricola* are based on Garita-Cambronero et al. (2017). The T3SS (-) strains of *X. campestris* and *X. arboricola* are all nonpathogenic, and the T3SS (+) strains are pathogenic, except *X. campestris* pv. *barbareae* CFBP 5825, which is considered nonpathogenic but it contains T3SS (Roux et al. 2015).

Xcc ATCC 33913

Xcr 756C

non-pathogenic Xan-nE1

Fig. 4. Schematic overview of the pathogenicity island (type III secretion system)-containing DNA fragment of *Xanthomonas campestris* pv. *campestris* 33913 (Xcc 33913) and *X. campestris* pv. *raphani* 756C (Xcr 756C) with the contig 11 of nonpathogenic *X. campestris* strain nE1 (Xan-nE1). The dashed lines refer to the DNA fragments of *X. campestris* pv. *campestris* 33913 and *X. campestris* pv. *raphani* 756c, 44.5 kb and 59.2 kb, which are absent in strain nE1. The pathogenicity island (black bar) from *hrpF* to *hpa2* is 23.1 kb in *X. campestris* pv. *campestris* 33913 and 37.8 kb in *X. campestris* pv. *raphani* 756c. Each arrow indicates the direction of transcription of a protein-coding gene, but its length does not correspond to the actual length of the gene. The genes on the flanking regions of pathogenicity island are indicated as 1 to 6 on the left region, and a to f on the right region. The genes with the same number or letter on the top are the same genes. For example, gene 6 refers to XCC1207, XCR_1446, and TR80_011000, which are all *mutS* (DNA mismatch repair protein) genes. The gene a refers to XCC1251, XCR_1506, and TR80_011015, which are the alpha-L-fucosidase (F5-8 type C domain protein) genes. The gene I and II indicate the genes present only in the *X. campestris* pv. *campestris* 33913 or strain nE1, respectively. The gene I of *X. campestris* pv. *campestris* 33913 is IS1478, and the gene II of strain nE1 is a chemotaxis protein gene (TR80_011005).

island, which is from XCR_1459 (*hrpF*) to XCR_1494 (*hpa2*), and also consists of five putative effector protein genes, XCR_1464 (*xopAD*), XCR_1464a (*xopAT*), XCR_1492 (*xopA*), XCR_1499 (*xopAL1*), and XCR_1500 (*xopP*).

The contig 11 (978 kb) of the strain nE1 can be mapped to the genes in the flanking regions of the above DNA fragment of *X. campestris* pv. *campestris* ATCC 33913 or *X. campestris* pv. *raphani* strain 756c. The contig contains the genes *mutS* (TR80_011000), corresponding to XCC1207 and XCR_1446, in the right border, and alpha-L-fucosidase (TR80_011005), similar to XCC1251 and XCR_1506, in the left border of the DNA fragments shown in Figure 4.

The absence of entire T3SS of strain nE1 is the reason why the strain did not amplify any DNA fragment in the *hrpF*-PCR. Besides, the assembled contigs of the strain nE1 contain none of 13 type III effector genes currently identified in *X. campestris* pv. *campestris* or *X. campestris* pv. *raphani* (Ryan et al. 2011; White et al. 2009), which include nine type III core effector genes, *avrBs2* (XCC0052), *xopN* (XCC0231), *xopR* (XCC0258, XCR_4254), *xopX* (XCC0529-0530), *xopQ* (XCC1072), *xopP* (XCC1247, XCR_1500), *xopZ* (XCC1975), *xopK* (XCC2899), and *xopL* (XCC4186), and four variable effector genes, *xopF1* (XCC1218, XCR_1470), *xopE2* (XCC1629), *xopAH* (XCC2109), and *xopD* (XCC2896). The *X. campestris* pv. *campestris* ATCC 33919 strain contains all of 13 effector genes, and *X. campestris* pv. *raphani* 756c strain has only three of them (*xopR*, *xopP*, and *xopF1*) (Ryan et al. 2011).

Although the strain nE1 does not contain the *hrp* gene cluster (pathogenicity island) and type III effector genes, the strains have the homologs of two transcriptional activator genes of pathogenicity island, *hrpG* and *hrpX* (both in the contig 9). Both *hrpG* and *hrpX* of strain nE1 share 96 to 97% identity with those of *X. campestris* pv. *campestris* ATCC 33913 and *X. campestris* pv. *raphani* 756c, but only have 77 to 85% identity with other species of *Xanthomonas*, such as *X. axonopodis* pv. *manihotis*, *X. vesicatoria*, *X. oryzae* pv. *oryzae*, and *X. citri*. The results further confirmed that the strain nE1 is indeed closely related to *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*.

PCR differentiation of *X. campestris* pv. *campestris*, *X. campestris* pv. *raphani*, and nonpathogenic *X. campestris* isolated from brassica seeds. Based on the above genome analyses, some genes can be utilized to design the primers to differentiate *X. campestris* pv. *campestris*, *X. campestris* pv. *raphani*, and nonpathogenic *X. campestris* by the PCR assay after the isolation of xanthomonads from brassica seeds by the Xan-D medium. Two transcription regulator genes, *hrpG* and *hrpX*, exist in all *X. campestris* pv. *campestris*, *X. campestris* pv. *raphani*, and nonpathogenic *X. campestris* strains, and the *hrpG*-PCR can be used to preliminarily determine whether the isolates belong to *X. campestris*

(Fig. 5A). The genes in the pathogenicity island, such as *hrpF*, are present in all *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* but not in the nonpathogenic *X. campestris*. The *hrpF*-PCR was employed to differentiate the pathogenic *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* from nonpathogenic *X. campestris* as in Figure 1B. As for the type III effector protein genes, *X. campestris* pv. *campestris* has more type III effector protein genes than *X. campestris* pv. *raphani*, and nonpathogenic *X. campestris* lack all of them. For example, *xopP* exists in both *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*, but the *avrBs2* is present only in *X. campestris* pv. *campestris* but not in *X. campestris* pv. *raphani*, which can be used to distinguish between *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* (Fig. 5B and C).

Selection of transconjugants and transfer of pathogenicity island. To determine whether the nonpathogenic *X. campestris* strain nE1 from the seeds can acquire the pathogenicity island from the endemic *X. campestris* pv. *campestris* or *X. campestris* pv. *raphani*, conjugation tests were undertaken. The identity of selected transconjugants of strain nE1 (Rif^R, Tc^R) was determined by PCR using the primer sets designed from the tetracycline resistance (Tc^R) gene of Tn5<TET-1> inside the *hrpF* of pathogenicity island in two donor bacteria, and two specific gene markers of strain nE1, *thuA* domain-containing protein (TR80_004955) and aldo/keto reductase family oxidoreductase (TR80_015515) genes (Fig. 6). The PCR-positive colonies were considered transconjugants of strain nE1. The strain nE1-MC1(Rif^R, Tc^R) transconjugant was selected from the mating between strain nE1 (Rif^R) and Xcc1-1 (*hrpF*::Tn5<TET-1>), and the nE1-MR1(Rif^R, Tc^R) transconjugant from the mating between the nE1(Rif^R) and Xcr-R1 (*hrpF*::Tn5<TET-1>).

Both nE1-MC1(Rif^R, Tc^R) and nE1-MR1(Rif^R, Tc^R) transconjugants acquired the *hrpF*::Tn5<TET-1> marker along with the pathogenicity island from Xcc1-1 (*hrpF*::Tn5<TET-1>) and Xcr-R1 (*hrpF*::Tn5<TET-1>) donor bacteria. The existence of pathogenicity island was determined by PCR assays using primer sets designed from the genes of pathogenicity island, such as *hrcC*, *hrcJ*, *hrcQ*, and *hrcR*. Two genes, *xopAT* and *xopAD*, only existing in the pathogenicity island of *X. campestris* pv. *raphani*, could be detected by the PCR assays only in nE1-MR1(Rif^R, Tc^R) but not in nE1-MC1(Rif^R, Tc^R) transconjugants. However, the type III effector protein genes outside the pathogenicity island, such as *xopD* and

Fig. 5. Differentiation of *Xanthomonas campestris* pv. *campestris* (Xcc), *X. campestris* pv. *raphani* (Xcr), and nonpathogenic *X. campestris* (nXan) strains by the PCR assay and agarose gel electrophoresis using the primer sets designed from **A**, the transcriptional regulator, *hrpG* (*hrpG*-F1/*hrpG*-R; 544 bp), and the type III effector genes, **B**, *xopP* (*xopP*-F/*xopP*-R; 954 bp) and **C**, *avrBs2* (*avrBs2*-F/*avrBs2*-R; 850 bp). Lanes 1 to 3: *X. campestris* pv. *campestris* strains Xcc1-1, Xcc-G, and Xcc-T1; lanes 4 to 6: *X. campestris* pv. *raphani* strains Xcr-X2B1, Xcr-X3A2, and Xcr-TGA; and lanes 7 to 9: nonpathogenic *X. campestris* strains nE1, nT1, and nA1. M, molecular size marker (1-kb Plus DNA ladder, Invitrogen Life Technologies, Carlsbad, CA).

Fig. 6. Determination of the identity of two transconjugants (T) of nonpathogenic *Xanthomonas campestris* strain nE1 selected from the conjugations between the pathogenic donor bacteria (D), *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*, and recipient bacterium (R), strain nE1 by the PCR assay and agarose gel electrophoresis using the primer sets designed from **A**, tetracycline resistance gene (Tc-F/Tc-R; 849-bp) of Tn5<TET-1> inside the *hrpF* of two donor bacteria, and two specific gene markers of strain nE1, **B**, *thuA* (TR80_004955), *thuA* domain-containing protein gene (*thuA*-F/*thuA*-R; 629-bp), and **C**, *akr* (TR80_015515), aldo/keto reductase family oxidoreductase gene (*akr*-F/*akr*-R; 659-bp). Lanes 1 and 2: the donor bacteria (D), *X. campestris* pv. *campestris* Xcc1-1 (*hrpF*::Tn5<TET-1>) and *X. campestris* pv. *raphani* Xcr-R1 (*hrpF*::Tn5<TET-1>); lane 3: the recipient bacterium (R), strain nE1 (Rif^R); and lanes 4 and 5: the transconjugants (T), nE1-MC1 (Rif^R, Tc^R) and nE1-MR1 (Rif^R, Tc^R). M, molecular size marker (1-kb Plus DNA ladder, Invitrogen Life Technologies, Carlsbad, CA).

avrBs2, were not detected in both transconjugants by PCR assays. The representative PCR results are shown in Figure 7. Even though nE1-MC1(Rif^R, Tc^R) and nE1-MR1(Rif^R, Tc^R) transconjugants acquired the pathogenicity island in which *hrpF* is knocked out, no type III effector gene was cotransferred.

DISCUSSION

This study provides the first experimental results indicating that brassica vegetable seeds, such as cabbage and cauliflower, could be infested with nonpathogenic *X. campestris*, *X. campestris* pv. *campestris*, or *X. campestris* pv. *raphani*, which cannot be distinguished by any semiselective media, such as the Xan-D medium. If nonpathogenic *X. campestris* cannot be differentiated from real pathogens, seed lots may be unnecessarily rejected or subjected to control treatments. The genomic and phylogenetic analyses in this study reveal that T3SS genes are important and useful genetic markers to differentiate nonpathogenic and pathogenic strains of *X. campestris*, which can be used to improve detection methods in the brassica seed health management.

Nonpathogenic xanthomonads have been isolated before from bean debris (Gilbertson et al. 1990), apple (Maas et al. 1985), tomato and pepper transplants (Gitaitis et al. 1987), rice seed lots (Ming et al. 1991), and *Prunus* spp. (Garita-Cambronero et al. 2017), and most nonpathogenic xanthomonads belong to *X. arboricola* and are not closely related to the pathovars of the plant from which they were isolated (Vauterin et al. 1996). In this study, *X. arboricola* was not found and the pathogenic and nonpathogenic xanthomonads isolated from the brassica seeds were all *X. campestris*. The nonpathogenic *X. campestris* were not assigned to any pathovar, because they are not pathogenic and the criterion of pathovar identification is based on host pathogenicity. Although nonpathogenic *X. campestris*, *X. campestris* pv. *campestris*, and *X. campestris* pv. *raphani* are closely related, they were not yet co-isolated from the same brassica seed lot in this study. It is possible that the number of nonpathogenic *X. campestris* overwhelmed those of pathogenic *X. campestris* on seed surface or vice versa, or that the examined colony number was not big enough to distinguish them. Alternatively, the closely related nonpathogenic and pathogenic *X. campestris* might compete with each other for the seeds (or even flowers and seed pots) as niches for survival. Therefore, it would be of interest to test whether the nonpathogenic xanthomonads could be used as a biological control agent to exclude *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* from brassica seeds and cause less disease incidence and severity during cultivation.

The MLSA analysis showed that the *X. campestris* and *X. arboricola* strains are first separated into two groups based on plant hosts, and each group was further divided into two subgroups according to the presence or absence of T3SS. The analysis provides the evolutionary sequence about the acquisition of pathogenicity in *Xanthomonas*. The nonpathogenic *X. campestris* strains isolated from brassica crops lack not only T3SS but also all type III effector genes and should be considered commensal bacteria, which have been first adapted to the brassica cultivation system, and inherit the capacities for epiphytic colonization on the hosts and saprophytic survival on the debris after harvest (Merda et al. 2017). The T3SS and type III effector genes may not be needed for them to live and multiply essentially in a dead-cell environment. Even the sugarcane pathogen *X. albilineans*, which lives in xylem (dead cells) limited lifestyle, also does not possess all genes for T3SS and any of the known type III effector genes (Pieretti et al. 2009).

As commensal bacteria, the nonpathogenic *X. campestris* strains contain two transcriptional activator genes, *hrpG* and *hrpX*, which also exist in pathogenic *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*. HrpG regulates the expression of *hrpX*, and HrpX can turn on genes especially for plant cell wall degradation, amino acid biosynthesis, and sugar and iron transport

(Wang et al. 2008). The genes for plant cell wall degradation include polygalacturonase, protease, and lipase genes, which belong to the HrpX regulon and are required for both pathogenic and saprophytic life processes. It is possible that whenever nonpathogenic or pathogenic *X. campestris* contacts plant tissue (dead or living), *hrpG* and *hrpX* could be induced to activate the genes for plant cell wall degradation, allowing the nonpathogenic *X. campestris* to obtain the nutrients from plant tissue surfaces or debris, or making the pathogenic *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* get access to the cell membranes of living cells. HrpX also activates the T3SS and type III effector genes in the pathogenic *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* in order to overcome the plant defense responses and cause the symptoms in the susceptible plant hosts (Huang et al. 2009; Wengelnik and Bonas 1996; Wengelnik et al. 1996).

All tested nonpathogenic *X. campestris* were negative in the *hrpF*-PCR and pathogenic *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* strains are all positive. The *hrp* genes have been used before to develop a PCR-based detection method to differentiate the pathogenic *X. campestris* pv. *vesicatoria* and *X. campestris* pv. *campestris* from nonpathogenic xanthomonads (Leite et al. 1994; Park et al. 2004). In this study, we provide direct genomic evidence that the negative result in the *hrpF*-PCR is attributed to the lack of the entire pathogenicity island (T3SS) in nonpathogenic *X. campestris*. Nonpathogenic *X. campestris* may acquire the pathogenicity island through horizontal gene transfer when the bacterium colonizes the same niche with pathogenic bacteria. Nonpathogenic *X. campestris* strain nE1 was demonstrated to be able to acquire the pathogenicity island by conjugation with two endemic brassica pathogens, Xcc1-1 and Xcr-R1, which were isolated from the diseased cabbage and radish in Taiwan. In a natural niche, it has been found that the chromosomal *hrp* or copper resistance genes could be indeed transferred by conjugation among pathogenic strains of *X. axonopodis* pv. *vesicatoria* (Basim et al. 1999). Therefore, it is possible that the nonpathogenic *X. campestris* from the imported seeds may have a chance to colonize the same niche with the endemic *X. campestris* pv. *campestris* or *X. campestris* pv. *raphani* in the brassica cultivation area, and the genetic material exchanges may take place between them to allow

Fig. 7. Determination of the existence of pathogenicity island (PI; type III secretion system) and type III effector genes in two transconjugants (T) of nonpathogenic *Xanthomonas campestris* strain nE1 selected from the conjugations between the pathogenic donor bacteria (D), *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*, and recipient bacterium (R), strain nE1 by the PCR assay and agarose gel electrophoresis using the primer sets designed from **A**, *hrcR* (*hrcR*-F+ *hrcR*-R; 463-bp), a gene of PI existing in *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*, **B**, *xopAT* (*xopAT*-F+*xopAT*-R; 325-bp), a gene of PI existing only in *X. campestris* pv. *raphani*, and **C**, *avrBs2* (*avrBs2*-F/*avrBs2*-R; 850-bp), a type III effector gene existing only in the *X. campestris* pv. *campestris*. Lanes 1 and 2: the donor bacteria (D), *X. campestris* pv. *campestris* Xcc1-1 (*hrpF*::Tn5<TET-1>) and *X. campestris* pv. *raphani* Xcr-R1 (*hrpF*::Tn5<TET-1>); lane 3: the recipient bacterium (R), strain nE1 (Rif^R); and lanes 4 and 5: the transconjugants (T) nE1-MC1 (Rif^R, Tc^R) and nE1-MR1 (Rif^R, Tc^R). M, molecular size marker (1-kb Plus DNA ladder, Invitrogen Life Technologies, Carlsbad, CA).

the nonpathogenic *X. campestris* to acquire the T3SS genes. Furthermore, type III effector genes are acquired afterward through additional gene transfers and accumulated stepwise in the non-pathogenic *X. campestris* as a consequence of differential adaptation to brassica host cultivars (Merda et al. 2017). It is also highly possible that the nonpathogenic *X. campestris* might transfer genes to *X. campestris* pv. *campestris* or *X. campestris* pv. *raphani* to enhance their survival capacities on the dead brassica leaves and/or on the seeds. Therefore, it cannot be ignored that nonpathogenic *X. campestris* on seeds could be a potential gene resource for horizontal gene transfer to cause the genetic diversity of the endemic pathogenic *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* strains.

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