Identification of Genes in *Xanthomonas euvesicatoria* pv. *rosa* That Are Host Limiting in Tomato

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Abstract: *Xanthomonas euvesicatoria* pv. *rosa* strain Xer07 causes a leaf spot on a *Rosa* sp. and is closely related to *X. euvesicatoria* pv. *euvesicatoria* (Xec) and *X. perforans* (Xp), causal agents of bacterial spot of tomato. However, Xer07 is not pathogenic on tomato and elicits a hypersensitive reaction (HR). We compared the genomes of the three bacterial species to identify the factors that limit Xer07 on tomato. Comparison of pathogenicity associated factors including the type III secretion systems identified two genes, xopA and xer3856, in Xer07 that have lower sequence homology in tomato pathogens. xer3856 is a homolog of genes in *X. citri* (xac3856) and *X. fuscans* pv. aurantifolii, both of which have been reported to elicit HRs in tomato. When xer3856 was expressed in *X. perforans* and infiltrated in tomato leaflets, the transconjugant elicited an HR and significantly reduced bacterial populations compared to the wildtype *X. perforans* strain. When xer3856 was mutated in Xer07, the mutant strain still triggered an HR in tomato leaflets. The second gene identified codes for type III secreted effector XopA, which contains a harpin domain that is distinct from the xopA homologs in Xec and Xp. The Xer07-xopA, when expressed in *X. perforans*, did not elicit an HR in tomato leaflets, but significantly reduced bacterial populations. This indicates that xopA and xer3856 genes in combination with an additional factor(s) limit Xer07 in tomato.

Keywords: *Xanthomonas euvesicatoria*; host associate factor; comparative genomics

1. Introduction

Florida is the leading producer of shrub roses in the U.S. [1]. A bacterial spot disease on rose (*Rosa* sp.) was reported in southern states including Florida and Texas in which *Xanthomonas* strains were isolated and shown to be pathogenic [2]. Multilocus sequence analysis and 16S rRNA sequences along with fatty acid profiles suggested that these rose pathogenic strains were highly similar to *X. alfalfae* subsp. *citriumelonis*. In a later study, Barak et al. [3] compared the whole genome sequence of GEV-Rose-07, one of the rose pathogenic strains, with other closely related *Xanthomonas* strains. Based on whole genome sequence analysis, GEV-Rose-07 was closely related to the taxonomic group Rademaker 9.2, which includes *X. euvesicatoria* (Xe) 85-10, *X. perforans* (Xp) 91-118, *X. euvesicatoria* pv. *allii* (Xea) CFBP 6369, *X. alfalfae* subsp. *citriumelonis* (Xac) F1, and *X. diefenbachiae* (Xd) LMG 12,749 [4]. With pairwise average nucleotide identity values >97% with *X. euvesicatoria*, GEV-Rose-07 was designated as a pathovar of *X. euvesicatoria*; *X. euvesicatoria* pv. *rosa* (Xer) [3].
Host range can tremendously vary among strains within a species due to variation in pathogenicity associated genes. Although taxonomically classified as *X. euvesicatoria* pv. *rosa* and closely related to *X. perforans* and *X. euvesicatoria* that cause bacterial spot disease on tomato and pepper, strain GEV-Rose-07, henceforth referred to as Xer07, is not pathogenic on either pepper or tomato, showing only minor chlorosis or HR-like symptoms at the inoculation site [2]. Due to its close relationship to strains infecting other hosts, identifying pathogenicity associated factors of strain Xer07 may improve understanding of the key factors influencing the host-pathogen interactions in this group of *Xanthomonas* and risk of host range expansion.

Host specificity, to a large extent, is dictated by delivery of type III secreted effectors (T3SEs) via the type III secretion system (T3SS) into host cells. T3SEs influence host-pathogen interactions by manipulating cellular activities [5]. In some cases, host plants carry resistance genes that recognize corresponding T3SEs (avirulence genes) and this interaction results in hypersensitive response (HR), characterized by rapid cell death to prevent bacterial spread [6,7]. For example, *X. perforans* and *X. euvesicatoria* contain effectors which limit their host range to tomato and/or pepper [8–10]. *X. perforans*, which does not infect *Nicotiana benthamiana*, contains two effectors, AvrBsT and XopQ, that when mutated allowed for host range expansion to *Nicotiana benthamiana* [10]. Barak et al. [3] computationally analyzed Xer07 for the presence, absence, or variation in effectors compared to *Xp* and *Xe* to identify potential host-limiting factor(s) in Xer07 and observed a stepwise erosion of T3SE genes in *X. euvesicatoria*, relative to the common ancestor of the group. However, they did not identify any obvious factors that would limit Xer07 pathogenicity in tomato and pepper.

In addition to T3SEs, other pathogenicity-associated factors in xanthomonads include genes encoding cell wall degrading enzymes such as endoxylanases, endoglucanases and pectate lyases [5,11]. Members of the glycosyl hydrolase (GH) family, XynA and XynB, were implicated in the reduction of virulence [12,13], and XynC also has been reported to contribute to bacterial virulence [14]. Likewise, Steffens et al. [15], determined that the lipopolysaccharide (LPS) synthesis clusters found in a *Xanthomonas translucens* pv. *translucens* elicited a stress response and were involved in pathogen signaling.

In the study by Barak et al. [3], analysis of T3SEs in several strains including the Xer07 strain that were placed in *X. euvesicatoria* did not identify any obvious factors that would limit Xer07 in tomato and pepper. Therefore, further comparative genomics are necessary to identify potential factor(s) in Xer07 that limit it from being pathogenic in tomato and pepper. The aim of this study was to identify host-limiting factors in Xer07 that prevent disease on tomato, despite Xer07 being closely related to tomato pathogens *X. perforans* and *X. euvesicatoria*. We identified two genes in Xer07 that, when expressed in Xp, successfully limited the ability of the bacterium to grow in tomato. Our results support a model in which host-specificity in *Xanthomonas* involves a complex interplay of multiple factors.

2. Results
2.1. Whole-Genome Sequence Similarity

The Xer07 genome was compared with 6 *Xanthomonas* species or pathovars available from the NCBI sequence database: *X. perforans* (36 strains), *X. euvesicatoria* (37 strains), *X. gardneri* (13 strains), *X. alfalfae* (1 strain), *X. axonopodis* (1 strain) and *X. vesicatoria* (7 strains) (Supplementary Table S1). Whole-genome sequence information of Xer07 along with some of the reference strains are listed in Table 1. Pairwise ANI comparisons based on Nucmer (Supplementary Table S1) showed Xer07 shared overall higher sequence similarities with strains of *X. perforans* (>99% sequence identity) and *X. euvesicatoria* (ANI between 98.7–99%). Following the study by Barak et al. [3], we further compared the pairwise ANI and in-silico DDH values within members of the Rademaker group 9.2 [2]. Both ANI and isDDH yielded consistent results in which Xer07 had slightly higher similarity values with *X. perforans* compared to *X. euvesicatoria* strains (Supplementary Table S1).
Table 1. Whole-genome sequence information of Xer07 along with reference strains.

| Strain                          | Host Name        | GenBank Accession No. | Total Sequence Length (bp) | GC Content (%) | Gene Count |
|---------------------------------|------------------|-----------------------|-----------------------------|----------------|------------|
| X. euvesicatoria pv. rosa GEV-Rose-07 | Rosa sp.         | GCA_001855615.1       | 4,970,862                   | 64.87          | 4510       |
| X. euvesicatoria pv. allii CFBP 6369 | Allium cepa (onion) | GCA_000730305.1       | 5,427,242                   | 64.35          | 4696       |
| X. alfalfae subsp. citrumelonis F1 | Rutaceae citrus (citrus) | GCA_000225915.1       | 4,967,469                   | 64.92          | 4188       |
| X. euvesicatoria LMG 12749     | Philodendron     | GCA_001401675.2       | 4,886,158                   | 64.91          | 4388       |
| X. perforans 91-118             | S. lycopersicum (tomato) | GCA_000192045.3       | 4,898,349                   | 65.04          | 4186       |
| X. euvesicatoria 85-10          | C. annuum (pepper) | GCA_000009165.1       | 5,420,152                   | 64.56          | 4707       |
| X. gardneri ATCC 19865          | Tomato           | GCA_000192065.2       | 5,528,124                   | 63.68          | 5424       |
| X. vesicatoria ATCC 35937       | Tomato           | GCA_000192025.2       | 5,531,089                   | 64.07          | 5117       |

2.2. Type III Secreted Effectors Repertoires

We identified 26 putative type III secreted effectors in the genome of Xer07 based on sequence homology with effectors found in other closely related xanthomonads (Table 2). Xer07 effector sequences were compared with representative strains of the closely related species Xanthomonas euvesicatoria pv. allii (Xea) CFBP 6369, X. alfalfae subsp. citrumelonis (Xac) F1, X. perforans (Xp) 91-118, and X. euvesicatoria (Xe) 85-10 (Table 2). Among the 26 effectors identified in Xer07, 22 of these effectors were present in all of the reference strains. Effector XopAE present in Xer07 was absent in Xe 85-10; XopAF was absent in Xe 85-10 and Xac F1; XopE2 was found in Xer07 and Xe 85-10; and XopAJ was found in Xer07, Xe 85-10, and Xac F1. Effectors found in multiple strains had high sequence homology, with overall amino acid identity above 95% except for XopA.

We further examined XopA due to its divergence from homologs in related strains. XopA in Xer07 is significantly different from Xp 91-118 (51%) and Xe 85-10 (50%) but identical to the XopA effector from Xac F1 and Xea CFBP6369. We aligned the amino acid sequence of XopA from additional representative genomes including pathogenic pepper strains Xe 85-10 and Xp 2010, pathogenic tomato strains Xp 91-118, Xp 17-12, Xp GEV904, and Xp GEV839, and other strains including Xe LMG12749, Xea CFBP6369, Xac F1, and Xoooc RS105. The xopA gene in Xer07 contains a distinct glutamine and glycine repeat region unlike Xe 85-10 and Xp 91-118 (Figure 1A) similar to the motif found in the harpin, HpaG [16,17]. Previous sequence comparisons of the HpaG and Hpa1 amino acid sequences from X. oryzae pv. oryzicola and X. oryzae pv. oryzae, respectively [18], identified two distinct domains that are conserved in many harpin-like proteins within Xanthomonas. The first region was required to prevent aggregation of the expressed proteins into inclusion bodies when the genes are expressed in E. coli, as deletion of the twelve amino acids (QGISEKQLDQLL) resulted in expression of insoluble proteins as inclusion bodies. The second region had no effect on inclusion body formation or elicitation of a hypersensitive response in tobacco leaves. Multiple sequence alignment of the amino acid sequences of XopA from Xp 91-118 and Xer07 with the amino acid sequences of HpaG and Hpa1 from X. oryzae pv. oryzicola and X. oryzae pv. oryzae (Figure 1B) show that all four proteins have the two domains, with 100% amino acid identity shared between all four proteins in region 1 and an 88.5% amino acid identity shared between all four species in the second domain. The presence of these canonical Xanthomonas harpin domains suggests that some other factor may be responsible for the difference in hypersensitive response in tomato observed between Xp 91-118 and Xer07.
Table 2. Putative type III secreted effectors in the genome of Xer07 and other xanthomonads including X. euvesicatoria 85-10, X. perforans 91-118, X. alfalfae subsp. citruminonis F1 and X. euvesicatoria pv. allii CFBP6369 based on sequence homology.

| Effectors | Synonyms | Xer07 | Xe 85-10 | Xp 91-118 | Xac F1 | Xea CFBP6369 |
|-----------|----------|-------|----------|-----------|--------|--------------|
| AvrBs2    | avrXac1  | +     | 704/714 (99%) 2 | 708/714 (99%) | 710/714 (99%) | 710/714 (99%) |
| XopE1     | avrXac2  | +     | 397/400 (99%) | 393/400 (99%) | 396/400 (99%) | 396/400 (99%) |
| HpaA      |          | +     | 277/278 (99%) | 272/275 (99%) | 271/275 (99%) | 271/275 (99%) |
| XopA      | Hpa1     | +     | 69/133 (52%) | 68/133 (51%) | 132/132 (100%) | 132/132 (100%) |
| XopAD     |          | +     | 510/530 (96%) | 530/530 (100%) | 463/531 (87%) | 461/530 (87%) |
| XopAE     | HpaF/G   | +     | 644/630 (97%) | 632/630 (97%) | 643/650 (99%) | 643/650 (99%) |
| XopAP     |          | +     | 417/423 (99%) | 424/427 (99%) | 419/423 (99%) | 419/423 (99%) |
| XopAU     |          | +     | 515/517 (99%) | 511/517 (99%) | 511/517 (99%) | 511/517 (99%) |
| XopAW     |          | +     | 216/221 (98%) | 220/221 (99%) | 221/221 (100%) | 221/221 (100%) |
| XopC2     |          | +     | 206/206 (100%) | 432/437 (99%) | 432/437 (99%) | 434/437 (99%) |
| XopF1     | Hpa4     | +     | 664/670 (99%) | 668/670 (99%) | 666/670 (99%) | 666/670 (99%) |
| XopF2     |          | +     | 653/667 (98%) | 660/667 (99%) | 645/671 (99%) | 645/671 (99%) |
| XopK      |          | +     | 630/634 (99%) | 614/634 (97%) | 614/635 (97%) | 614/635 (97%) |
| XopL      |          | +     | 634/661 (99%) | 633/661 (99%) | 639/661 (97%) | 639/661 (97%) |
| XopN      |          | +     | 718/733 (99%) | 724/733 (99%) | 728/733 (99%) | 728/733 (99%) |
| XopQ      |          | +     | 460/464 (99%) | 459/464 (99%) | 456/464 (98%) | 456/464 (98%) |
| XopR      |          | +     | 399/404 (99%) | 401/404 (99%) | 399/404 (99%) | 399/404 (99%) |
| XopS      |          | +     | 301/308 (98%) | 305/308 (97%) | 307/308 (98%) | 307/308 (98%) |
| XopV      |          | +     | 338/346 (98%) | 341/346 (98%) | 342/346 (98%) | 342/346 (98%) |
| XopX      |          | +     | 688/721 (98%) | 680/714 (95%) | 685/721 (95%) | 685/721 (95%) |
| XopZ      |          | +     | 137/1388 (99%) | 1372/1388 (99%) | 1377/1388 (99%) | 1377/1388 (99%) |
| XopP      |          | +     | 567/577 (98%) | 617/627 (98%) | 629/641 (98%) | 629/641 (98%) |
| XopAF     | avrXv3   | +     | 217/218 (99%) | 444/450 (98%) | 444/450 (98%) | 443/450 (98%) |
| XopI      |          | +     | 443/450 (98%) | 217/218 (99%) | 217/218 (99%) | 217/218 (99%) |
| XopE2     | avrXacE3 | +     | 352/358 (98%) | – | – | – |
| XopAJ     | avrXs01  | +     | 412/421 (98%) | – | 321/324 (98%) | – |

1 Symbol “+” indicates presence of type III effector in Xer07; 2 Type III effector nucleotide sequence identity of each isolate when compared to Xer07; 3 Symbol “−” indicates the absence of type III effector in respective isolates; 4 Symbol “&” indicates the type III effector distributed in two different contigs.

Figure 1. Comparison of XopA and Xer3856 amino acid sequences in Xer07 with sequences in other xanthomonads including X. alfalfae subsp. citruminonis F1, X. euvesicatoria pv. allii CFBP6369, X. euvesicatoria 85-10, X. perforans 91-118 and X. perforans GEV904, X. perforans Xp17-12, X. perforans GEV839, X. perforans Xp2010, X. euvesicatoria LMG12749, X. oryzae pv. oryzicola RS105. (A) xopA gene missing distinct harpin motif (depicted by dashes) in X. euvesicatoria (85-10) and X. perforans (91-118) but present in X. euvesicatoria (Xer07). (B) xer3856 gene found in Xer 07.
We determined that 16 effectors were absent in Xer07 but present in either Xe or Xp (Table 2). AvrBs1, AvrRxv, XopAA, HolPsyAE, XopAV, XopAX, XopB, XopC, XopG, XopH, and XopO were found in Xe 85-10, while XopAK, XopJ4 and an ortholog of XopX were present in strain Xp 91-118. Sequence length of XopAK varied within all of the strains that were compared to Xer07, missing a significant coding region with only 142 amino acids out of 485, suggesting that the gene may be non-functional in Xer07. Interestingly, the same copy of this gene in addition to the XopAK effector was found in strain Xp 91-118. XopD, a SUMO protease effector [19], that is present in both Xp 91-118 and Xe 85-10 is also absent in Xer07 (Table 2).

2.3. xer3856 as Host-Limiting Candidate

A homolog of Xac3856 in X. citri and a homolog in X. fuscans pv. aurantifolii (Xfa) designated Xfa3856 were identified in Xer07 and designated as Xer3856 (Figure 1B, Supplementary Figure S1). This was considered a potential host-limiting factor, since the Xfa3856 was shown to induce a hypersensitive response in tomato independent of type III secretion system, whereas homologs present in tomato strains do not elicit an HR [20]. Homologs of xac3856 and xfa3856 were found to be present in all strains used in this study. However, the sequences varied significantly in length (Figure 1B). Strains infecting pepper (Xe 85-10, Xp 2010) and tomato (Xp 91-118, Xp GEV904, Xp 17-12, Xp GEV839) were missing 63 amino acids compared to Xer07-xer3856. Rather interestingly, the Xer07-xer3856 was over 99% identical to genes found in both Xac F1 from citrus and Xe LMG12749 from Philodendron.

2.4. XopA and Xer3856 Are Host-Limiting Factors in Tomato

Given that comparative genomics indicated XopA and Xer3856 were potential candidates for limiting the ability of Xer07 to infect tomato, the two genes were cloned from Xer07 and expressed in tomato strain Xp 91-118 to evaluate their role in limiting growth in tomato. xopA and xer3856 clones were conjugated into tomato pathogenic strain Xp 91-118 via triparental mating to generate Xp 91-118::Xer07-xopA and Xp 91-118::Xer07-xer3856. Bacterial suspensions of transconjugants adjusted to 10^8 CFU/mL were infiltrated into the mesophyll of susceptible cultivar Bonny Best along with the wildtype Xp 91-118. Xp 91-118::Xer07-xer3856 induced a strong HR within 36 h post inoculation (Figure 2A) but Xp 91-118::Xer07-xopA didn’t induce a visible HR (Figure 2B). Simultaneously, the strains were also infiltrated at concentrations of 10^5 CFU/mL in tomato to determine bacterial population over time. The results showed that the Xp 91-118::Xer07-xopA and the Xp 91-118::xer3856 populations were significantly lower than those in the wildtype Xp 91-118 strain (Figure 2C,D).

In order to determine if inactivation of xer3856 eliminates an HR when Xer07 is infiltrated in tomato, the gene was mutated using pCR2.1TOPO-vector from Xer07 to generate the insertion mutant Xer07 Ωxer3856 (Table 3). Both Xer07 and Xer07 Ωxer3856 induced an HR in tomato (Figure 3A). Furthermore, the Xer07 Ωxer3856, Xer07 and Xp 91-118::pUFR strains were infiltrated into leaflets of Bonny Best tomato cultivar to quantify bacterial populations. Xp 91-118 populations were ~2 log CFU/mL higher than Xer07 Ωxer3856 and wildtype Xer07 over the sampling period following infiltration (Figure 3B).
Hypersensitive reaction and population dynamics following infiltration of Bonny Best tomato leaflets. Infiltration of suspensions of (A) Xp 91-118::Xer07-xer3856 and (B) Xp 91-118::Xer-xopA in tomato at \( \sim 5 \times 10^8 \) CFU/mL concentration. Note HR in leaflet infiltrated with Xp 91-118::xer3856 but not Xp 91-118::Xer-xopA (C) Bacterial populations of Xp 91-118::pUFR, wildtype Xer07, and Xp 91-118::xer-3856, and (D) Population of Xp 91-118::pUFR, wildtype Xer07, and Xp 91-118::Xer-xopA following infiltration with suspensions adjusted to \( \sim 10^5 \) CFU/mL at \( \sim 10^5 \) CFU/mL in tomato.

**Figure 2.** Hypersensitive reaction and population dynamics following infiltration of Bonny Best tomato leaflets. (A) Hypersensitive reactions on tomato following infiltration with Xer07 Ωxer3856, Xer07, and Xp 2010 (a pepper isolate susceptible on tomato) at concentration of \( \sim 5 \times 10^8 \) CFU/mL. HR was observed 24 h post inoculation with Xer07 Ωxer3856 and Xer07 no no significant damage was observed in leaflet infiltrated with Xp 2010. (B) Population dynamics of Xer07 Ωxer3856, Xer07 and Xp 91-118::pUFR in tomato leaflets at various times after infiltration of bacterial suspension at concentration of \( 10^5 \) CFU/mL. Populations were significantly lower for Xer07 Ωxer3856 and Xer07 in comparison with Xp 91-118::pUFR. Vertical lines indicate standard error.

**Figure 3.** Hypersensitive reaction and population dynamics following infiltration of Bonny Best tomato leaflets.
Table 3. List of strains used in the study.

| Strain       | Characteristics                  | Source                      |
|--------------|----------------------------------|-----------------------------|
| Xer07        | Xanthomonas strain isolated from Rose | This study                  |
| Xp 91-118    | X. perforans isolated from tomato | This study                  |
| Xe 85-10     | X. euvesicatoria isolated from pepper | This study                  |
| E. coli DH5α | Competent cell for hosting the plasmid | Bethesda Research Laboratories |

| Strain       | Characteristics                  | Source                      |
|--------------|----------------------------------|-----------------------------|
| Xer07Ωxer3856| Xer07, xer3856 mutated using TOPO, KanR | This study                  |
| Xp 91-118::xer3856| Xp 91-118 complemented with xer3856, KanR | This study                  |
| Xp 91-118::xopA| Xp 91-118, complemented with xopA from Xer 07, KanR | This study                  |

Plasmids

| Plasmid       | Characteristics                               | Source                      |
|---------------|-----------------------------------------------|-----------------------------|
| pCR2.1-TOPO   | TOPO plasmid, KanR                            | Thermofisher                |
| pCR2.1-TOPO-xer3856| PCR product of xer3856-int-F and xer3856-int-R into pCR2.1-TOPO, KanR | This study                  |
| pUFRO34       | Cloning vector, KanR                          | This study                  |
| pUFRO34-xer3856| PCR product of xer3856-out-F and xer3856-out-R into pUFRO34, KanR | This study                  |
| pUFRO34-xopA  | PCR product of xopA-F and xopA-R from Xer07 into pUFRO34, KanR | This study                  |
| pGEM-T Easy   | Cloning vector, AmpR                          | Promega                     |
| pGEM-T Easy-xer3856| xer3856 in pGEM-T for cloning, AmpR            | This study                  |
| pGEM-T Easy-xopA| xopA from Xer07 in pGEM-T for cloning to pUFRO34-xopA, AmpR | This study                  |

2.5. Structure of XopA

XopA found in Xer07 carried an additional Glutamine and Glycine repeat region different from the X. perforans and X. euvesicatoria strains that are pathogenic on tomato. Four different protein structure prediction models from Phyre2, RaptorX, iTasser, and trRosetta were used to evaluate the possible structural differences between the XopA proteins found in Xer07 and X. perforans 91-118. The Phyre2 server could only model 6% (8 amino acids) of the submitted sequence for the Xer07 XopA protein with 28% confidence and 14% (16 amino acids) with 22% confidence of the Xp 91-118 XopA protein. The two other homology modeling algorithms used, RaptorX and iTasser, varied widely in the models returned for each protein, with each model of each protein having a completely different predicted tertiary structure than the other model created for the same input sequence. This is not surprising as harpin proteins vary widely in their amino acid sequence and localization, either extracellularly or as an effector protein injected into the host cell via the T3SS [18,21]. Superpositions of the models generated by RaptorX (Figure 4A) show two alpha helices that are superposable, the first spanning amino acids 36-54 in Xp 91-118 (37-52 in Xer07), and the second spanning amino acids 37-52 in Xp 91-118 (88-103 in Xer07). None of the remaining amino acids are superposable, in large part due to the fifteen amino acid deletion in the Xp 91-118 XopA protein. In XopA from Xer07 and Xoo, an amino acid sequence of QGQGDSGGQGNSQ is present (Figure 5), resulting in an extended loop being formed between amino acids 59 and 86 (Figure 4B). Since the proteins from all four organisms contain the two regions demonstrated to be necessary for function [18], we hypothesize that this additional loop in the Xer07 XopA protein adopts an alpha-helical secondary structure when in the presence of its cognate binding partner, much the same as is seen in the X. oryzae pv. oryzae and X. oryzae pv. oryzicola homologs. The formation of a complex between the Xer07 XopA protein and a plant protein may be the reason why Xer07 XopA homolog is capable of eliciting a defense response in tomato, but Xp 91-118 XopA is not.
2.5. Structure of XopA
XopA found in Xer07 carried an additional section with a 98% similarity compared to XopA from X. perforans (91-118) (Tan). Superposition of the lowest energy homology models of X. perforans (91-118) (Tan) and Xer07 (blue) created by trRosetta.

2.6. Comparative Genomics of Other Pathogenicity Associated Factors
We compared the genes for proteins secreted by the type II secretion system that are present in X. oryzae pv. oryzicola and the Hpa1 gene from X. oryzae pv. oryzae. The two regions previously shown to be conserved between the X. oryzae pathovars and that are found in X. perforans 91-118 and Xer07 are shown as is the deleted region (red box) in the X. perforans 91-118 sequence.

Figure 4. (A) Superposition of the lowest energy homology models of XopA from X. perforans (91-118) (Tan) and Xer07 (blue). The amino and carboxy-terminal residues of each protein are labelled. (B) Superposition of the lowest energy de novo models of X. perforans (91-118) (Tan) and Xer07 (blue) created by trRosetta.

Figure 5. Sequence alignment of the XopA genes from X. perforans 91-118, Xer07, the HpaG gene from X. oryzae pv. oryzicola and the Hpa1 gene from X. oryzae pv. oryzae. The two regions previously shown to be conserved between the X. oryzae pathovars and that are found in X. perforans 91-118 and Xer07 are shown as is the deleted region (red box) in the X. perforans 91-118 sequence.
3. Discussion

We compared genes involved in pathogenicity and host range in related xanthomonads from pepper, tomato, and other host species to those in strain Xer07 from rose. Xer07 is closely related to X. perforans based on ANI and in silico DDH, but causes an HR in tomato, the primary host of X. perforans. We identified two host associated factors in X. euvesicatoria pv. rosa that limited its ability to cause disease on tomato. Our results further confirmed that Xanthomonas taxonomy is not driven by host range and showed that differences in multiple specific pathogenicity factors among phylogenetically similar strains can alter host specificity.

We identified two genes in Xer07 that contributed to limiting infection in tomato. The first gene, xer3856, a homolog of genes in X. citri and X. fuscans pv. aurantifolii was identified in Xer07. In two studies, xfa3856, when expressed in the tomato pathogen Xp 91-118 and infiltrated in tomato leaves resulted in elicitation of an HR [20,22]. Xfa3856 homologs are also present in X. euvesicatoria and X. perforans with high sequence similarity, although the latter two homologs encode for a truncated protein. Xfa3856 is predicted to have a putative transmembrane helix and two EF-hand calcium binding motifs at the C-terminus [20]. When xer3856 was cloned and expressed in Xp 91-118, the transconjugant induced an HR in tomato plants. However, the mutated version, Xer07 Δxer3856, performed similarly as wild-type Xer07, and still triggered HR in tomato. These results indicated that xer3856 from Xer07 elicits a resistant reaction in tomato and is one of multiple host limiting factors for Xer07 in tomatoes.

The type III secretion system is crucial for pathogenic Xanthomonas to colonize plants and to translocate type III effectors to interfere with cellular functions. We identified 26 potential type III effectors/Xanthomonas outer proteins (Xops) present in Xer07. XopA is the name designated for the Hpa1 protein that contains harpin-like protein and is secreted through T3SS transfer [23]. Interestingly, XopA homologs were present in all Xanthomonas strains in this study. XopA was found to be necessary for strain Xe 85-10 to grow in planta and maintain full virulence [24]. Deletion of XopA delayed an HR and water-soaking symptom and reduced bacterial growth in pepper leaves compared to wild-type Xe 85-10. In this study, we observed that the xopA gene from strains pathogenic to different hosts varied in sequence. Tomato and pepper pathogenic strains, including Xp 91-118, Xe 85-10, Xp 17-12, Xp GEV904, Xp GEV839, and Xp 2010, shared only ~50% amino acid identity in XopA with Xer07, Xae F1 and CFBP 6369. Similarity among species may be ancestral (trans-specific variation) or the result of recombination event within or including the gene. A study by [25], showed that HpaG (also referred as Hpa1) with the feature of harpins, was able to elicit an HR in pepper but not in tomato plants, and Hpa1 from X. oryzae pv. oryzae induced HR in tobacco plants. A recent study also confirmed that the expression of XopA from X. oryzae pv. oryzicola RS105 in N. benthamiana was able to trigger HR symptoms [26]. We expressed XopA from Xer07 in Xp 91-118 to evaluate if it can independently act as a host limiting factor. Interestingly, the bacterial populations of Xp 91-118::XopA were reduced by more than 2 log-folds compared to the Xp 91-118::pUFR (Empty vector).

In addition to their individual role as an effector, several possibilities have been studied to gain insights into the function of harpin-type bacterial proteins. The XopA/Hpa1-like effectors have been found to play a significant role in translocation of associated effectors [27]. Wang et al. [18], demonstrated that Hpa1 is a type III translocator which is critical for translocation of two transcription activation like (TAL) effectors PthXo1 and AvrXa10 that affect the phenotype in susceptible and resistant genotypes. Although the XopA harpin in Xer07 when expressed in X. perforans resulted in a reduction in bacterial populations in tomato, the reason remains to be determined. In other studies, XopA homologs in transconjugants expressing an extra copy of HrpG were shown to elicit an HR in tobacco or citrus [25,28]. Kim et al. [25], determined that X. axonopodis pv. glycines expressing extra copies of HrpG also overexpressed hpa1 which was associated with elicitation of an HR in tobacco leaves. In this study there may have been a slight increase in expression of xopA, although not to the level observed in transconjugants expressing multiple copies of HrpG.
The results confirm multiple factors can limit pathogenicity towards a host and can either induce a phenotypic reaction as Xer3856 or limit the population growth as XopA. The Glutamine and Glycine repeat region found in the XopA of Xer07 was similar to a motif, motif 2 found in the HpaG harpin protein from X. axonopodis pv. glycines. The motif 2 region of HpaG is homologous to the prion-forming domain of the yeast prion protein Rnq1p [16] and HpaG secretion was shown to induce HR in plants by formation of amyloid-like fibrils [17]. Among the three motifs described in HpaG from X. axonopodis pv. glycines, homologs to motifs 1 and 3 were found in other genomes compared in this study. We identified differences in the effector repertoires of Xer07, X. cuyesecatoria, X. perforans, and other closely related species that could be explored in future studies. As an example, XopD is absent from Xer07 but present in both X. cuyesecatoria and X. perforans strains. Based on protein analysis of XopD, it was shown to contain a small ubiquitin-like modifier (SUMO) protease domain that belongs to the C48 protease family as reversible post-translational modifiers [29]. Mutation of xopD gene in Xe 85-10 followed by inoculation resulted in plants exhibiting severe chlorosis and tissue necrosis and increased salicylic acid levels compared to wild-type, which suggested XopD’s ability to delay symptom progression and function as a tolerance-promoting factor [19,23]. It is apparent that Xer07 strain expressing XopD should be created to determine whether XopD is linked to virulence on tomato and pepper. However, this can only be evaluated once we can successfully eliminate the HR phenotype that restricts Xer07 in tomato. Additionally, the absence of XopD and 11 other effectors that were found in Xe 85-10 and Xp 91-118 could be other potential genes that could influence Xer07 pathogenicity in different hosts.

Among the two type II secretion systems found in many xanthomonads, T2SS-xps is conserved in all Xanthomonas spp. [5]. In a study by Szczesny et al. [14], the xps system was shown to be required for extracellular protease and xylanase activity, as deletion of the xps but not xcs in Xe 85-10 significantly reduced halo formation when incubated on NYG plates containing milk or xylan. Sequence identity of DSF cell-cell signaling system and the arrangement and content of xylanolytic enzyme clusters were found to be conserved among the Rademaker group 9.2 strains that encompasses the Xer07, compared to Xv and Xg strains. Meanwhile the xylanase genes xynC/xyn5A were not present in Xv ATCC 35937 and Xg ATCC 19,865 but in the Rademaker group 9.2 strains. A deletion of xynC in Xe 85-10 caused a reduction in bacterial growth in planta suggesting xynC/xyn5A is an active xylanase and can contribute to virulence [14]. Similar high sequence and cluster similarity was found in LPS gene cluster among the Xer07 and Xe 85-10 that was distinct from the X. perforans strain 91-118. Potnis et al. [11] predicted a putative horizontal gene transfer event resulting in the acquisition of novel LPS gene cluster in X. perforans that may have played a major role in X. perforans specificity in tomato.

In this comparative study we demonstrate that Xer07 is closely related to Xp and Xe. As we focused on identifying host limiting factors in GEV-Rose-07, we successfully demonstrated that xer3856 gene induced an HR in tomato and XopA from Xer07 significantly limited bacterial growth in tomato. Recognition of these host-limiting factors in Xer07 improves our knowledge in host pathogen interactions of Xp and Xe on tomato and pathogen host range evolution that can be used to design durable resistance mechanisms in plant hosts.

4. Material and Methods
4.1. Bacterial Strains and Growth Conditions

Bacterial strains that were used for assaying pathogenicity and quantifying internal bacterial population dynamics included Xer GEV-Rose-07 strain (pathogenic on Rosa spp.), Xe E3 (pathogenic to pepper), Xe 85-10 (pathogenic to pepper), and Xp 91-118 (pathogenic to tomato) (Table 3). The strains were stored at −80 °C in 30% glycerol for long term storage. Fresh cultures used in this study were obtained by streaking the bacterium on nutrient agar (NA) plates followed by incubation at 28 °C for 48 h. Individual colonies were then streaked on NA plates and incubated at 28 °C for 24 h for use during experiments. For
**Escherichia coli** strains used during mutant constructions, the plates were incubated at 37 °C. The list of strains and plasmid constructs are listed in Table 3.

### 4.2. Genome Collection and Genome Statistics

Representative complete and draft genome sequences of *Xanthomonas* spp. were obtained from GenBank database (Supplementary Table S1). The assembled genomes were compared with pairwise average nucleotide identity (ANI) analysis and in-silico DNA-DNA hybridization (isDDH) analysis based on genome-to-genome comparisons [30,31]. The pairwise ANI values were obtained from nucmer (NUCleotide MUMmer). Similarly, isDDH was estimated using the Genome-to-Genome Distance Calculator (GGDC) 2.0 Web server (http://ggdc.dsmz.de/distcalc2.php) (accessed on 15 March 2019).

### 4.3. Effector Repertoire and Pathogenicity Associated Genes

With the objective of identifying host specificity factors in *Xer07*, the annotated sequences from IMG/JGI were downloaded and searched by BLAST analysis for effectors using a list of 81 type III effectors compiled from different *Xanthomonas* species (Potnis and Iruegas-Bocardo, personal communication; www.xanthomonas.org (accessed on 9 May 2018)). Effector sequences were extracted based on amino acid sequence homology using local BLAST [32]. Effector with more than 70% sequence homology compared with the reference was considered as being present. The sequences for the effectors predicted for *Xer07* were further evaluated manually and compared with annotations from IMG/JGI to confirm their presence. The *xer3856* gene and its homology to the *xfa3856* was determined based on BLAST comparisons.

### 4.4. Mutants, Transconjugants and Population Dynamics

The *xer3856* gene was mutated in *Xer07* using pCR2.1-TOPO vector (TOPO® TA Cloning Kit, invitrogen™ [33]) to generate *Xer07*Δ*xer3856*. In order to determine if *xer3856* is the factor responsible for eliciting an HR in tomato and pepper, we infiltrated Bonny Best tomato leaflets with suspensions adjusted to ~10^8 CFU/mL of strains *Xer07*Δ*xer3856*, and *Xer07* along with the tomato pathogen, *X. perforans*, *Xp* 91-118. Additionally, the bacterial populations of *Xer07*Δ*xer3856*, *Xer07* and *Xp* 91-118 were evaluated in tomato by quantifying bacterial growth as described above. In order to independently evaluate the role of *Xer3856* and XopA in tomato pathogenicity, plasmids carrying these individual genes were conjugated into *Xp* 91-118. To create these plasmids, the genes *xopA* and *xer3856* were amplified using primers as specified in Supplementary Table S4. The amplicon was cloned with pGEM-T easy vector and subsequently excised from pGEM-T vector and ligated into pUFR034 and mobilized into *Xp* 91-118 through triparental mating for in planta analysis.

### 4.5. Comparative Genomics

In addition to the type III secreted effectors, additional pathogenicity factors were compared between *Xer07*, *Xp* 91-118, *Xr* 85-10, *Xac* F1, *Xea* CFBP6369, *X. gardneri* ATCC19865 (*Xg* ATCC19865) and *X. vesicatoria* ATCC35937 (*Xv* ATCC35937). Type III secretion system cluster, cell-wall degrading enzyme cluster, lipopolysaccharide biosynthetic clusters and diffusible signal factors that are considered important for bacterial virulence were compared among the four closely related strains. The reference genes and their homologs were identified using BLAST and homology search was carried out using the IMG/JGI online platform (www.img.jgi.doe.gov) (accessed on 1 April 2019).

### 4.6. Pathogenicity Assay

In order to evaluate pathogenicity/resistance in tomato, bacterial strains used in the study were inoculated at variable concentrations. Bacterial inoculum was adjusted to 10^8 CFU/mL (*A_600* = ~0.3 at) and infiltrated with a hypodermic needle syringe into Bonny Best tomato leaflets to determine HR. Plants were placed in growth chambers at 28 °C and the infiltrated area was observed for HR or susceptible reaction. HR was confirmed
by the presence of confluent necrosis in infiltrated area due to rapid cell death, 24 to 48 h post inoculation.

Bacterial populations were also determined in Bonny Best tomato. Bacterial suspensions at \( \sim 10^5 \) CFU/mL were infiltrated into the leaflets and plants were placed in growth chamber at 28 °C. Inoculated leaf tissue was sampled every 48 h for 10 days. A 1-cm² leaf disk was sampled from each leaflet and the tissue was ground in sterile tap water and the resulting suspensions were serially diluted between \( 10^{-1} \)–\( 10^{-5} \) fold. Fifty-microliters from the suspensions were plated on NA and the plates were incubated at 28 °C. The assay was replicated three times for determining bacterial populations in planta.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11060796/s1, Supplementary Figure S1: Open reading writing—original draft, Q.F., S.B., J.C.H., J.B.J., and S.T.; writing—review and editing, Q.F., S.B., A.E.V., G.E.V., E.M.G., J.C.H., J.B.J. and S.T. All authors have read and agreed to the published version of the manuscript.

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