Xanthomonas oryzae (Xo) are globally important rice pathogens. Virulent lineages from Africa and Asia and less virulent strains from the United States have been well characterized. Xanthomonas campestris pv. leersiae (Xcl), first described in 1957, causes bacterial streak on the perennial grass, Leersia hexandra, and is a close relative of Xo. L. hexandra, a member of the Poaceae, is highly similar to rice phylogenetically, is globally ubiquitous around rice paddies, and is a reservoir of pathogenic Xo. We used long read, single molecule real time (SMRT) genome sequences of five strains of Xcl from Burkina Faso, China, Mali, and Uganda to determine the genetic relatedness of this organism with Xo. Novel transcription activator-like effectors (TALEs) were discovered in all five strains of Xcl. Predicted TALE target sequences were identified in the Leersia perrieri genome and compared to rice susceptibility gene homologs. Pathogenicity screening on L. hexandra and diverse rice cultivars confirmed that Xcl are able to colonize rice and produce weak but not progressive symptoms. Overall, based on average nucleotide identity (ANI), type III (T3) effector repertoires, and disease phenotype, we propose to rename Xcl to X. oryzae pv. leersiae (Xol) and use this parallel system to improve understanding of the evolution of bacterial pathogenicity in rice agroecosystems.

Keywords: Xanthomonas oryzae, transcription activator-like effectors (TALEs), agroecosystem, cutgrass, rice
INTRODUCTION

Rice is a staple crop for more than half the world. Severe rice diseases, such as bacterial leaf streak (BLS) caused by *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) and bacterial blight (BB), caused by *X. oryzae* (*Xo*), are increasing in prevalence in parts of Asia and sub-Saharan Africa and cause significant yield losses. In Asia, perennial weeds are considered an important source of primary pathogen inoculum for these two diseases (Ou, 1985; Mew, 1987).

Southern cutgrass (*Leersia hexandra* Swartz) is a common grass found in the southern United States, South America, Africa, and Asia. It is a member of the Poaceae family and is closely related to rice, but diverged from *Oryza* approximately 14 mya (Guo and Ge, 2005). *L. hexandra* is an invasive species that frequently grows along rivers and canals surrounding rice paddies. Because of its close relationship to *Oryza* spp., *Leersia* spp. are included as outgroups in phylogenetic studies. Recent genome investigations of *Leersia perrieri*, a cutgrass found in Madagascar, were done to compare repetitive elements and transposable elements among *Oryza* sp. and to uncover orthologs of the important submergence tolerance gene, *SUB1* (Copetti, 2013; Copetti et al., 2015; dos Santos et al., 2017). One-third of the *L. perrieri* genome was found to consist of repeats. The high amount of newly discovered repeats (35%) indicates that the *L. perrieri* genome is evolving rapidly relative to the *Oryza* genus (Copetti, 2013).

Early reports of phytoremediation by *L. hexandra* showed this grass’ capacity to sequester Cu, Cr, and Ni, and it has now been proposed as a tool in wastewater treatment (Liu et al., 2011; Wang et al., 2012; You et al., 2013). Interestingly, this and other grasses in this genus, such as *Leersia sayanuka*, *Leersia oryzoides*, and *Leersia japonica*, are susceptible to *Xoo* (Noda and Yamamoto, 2008) and can serve as reservoirs for inoculum. *Xoo* strains isolated from symptomless *L. hexandra* cause BB symptoms in rice, and, in artificially inoculated weed plants, *Xoo* multiplied without evidence of disease (Gonzalez et al., 1991) implicating this grass as an alternative host for the pathogen. These and other findings reinforce that effective integrated management of crop diseases must incorporate knowledge of pathogen interactions with weedy species.

The species *Xo* is highly diverse, and is represented by distinct lineages of *Xoo* from Asia and Africa, *Xoc* from Asia and Africa, and strains not assigned as a pathovar from the United States, *Xo* (Gonzalez et al., 2007; Triplett et al., 2011; Hajar et al., 2012; Poulin et al., 2015; Triplett and Leach, 2016). The term pathovar is used to refer to a strain or set of strains with the same or similar characteristics, differentiated at infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts1. Poulin et al. (2015) used multi-locus variable-number tandem-repeat analysis (MLVA) to investigate genetic structures of microbial populations (Zhao et al., 2012; Poulin et al., 2015), and suggested that *Xoo* and *Xoc* from Africa had a common Asian ancestor; this conclusion was based on the fact that the allelic richness, or number of alleles, was significantly less in these populations. However, further analyses on an extensively sampled set of isolates are needed to confirm this ancestral hypothesis.

*Xanthomonas* spp. inject effector proteins into plant host cells to elicit disease via a type-III (T3) secretion system (White et al., 2009). These proteins can confer pathogenicity and/or dictate host specificity (Jacques et al., 2016). *Xanthomonas* spp. are most notable for production of transcription activator-like effectors (TALEs). TALEs influence host gene expression by directly binding to specific sequences [effector binding elements (EBEs)] in the target promoter as dictated by repeat-variable di-residues (RVDs) at the 12 and 13 amino acid position in the central repeat region (CRR) (Boch et al., 2009; Moscou and Bogdanove, 2009; White et al., 2009; Bogdanove and Voytas, 2011). The CRR contains different numbers of repeats, each with 33–35 amino acids. TALEs may enhance diseases by targeting susceptibility (S) genes, or may trigger a resistance response through activation of an “executor” resistance gene (R) expression (Boch et al., 2014; Hutin et al., 2015).

The presence of TALE effectors is variable in the genus (Jacques et al., 2016). *Xoo* contain nine to 20 TALEs while *Xoc* can contain up to 29. US *Xo* do not contain any TALEs, and due to this absence, have been employed as a tool to study TALE effector biology in rice (Ryba-White et al., 1995; Verdict et al., 2012). New sequencing technologies and predictive algorithms have accelerated the characterization of TALEs and their host gene targets. In particular, long read, single molecule real time (SMRT) sequencing (Pacific Biosciences, Menlo Park, CA, United States) has enabled the rapid assembly of TALE sequences that are otherwise laborious to capture due to their highly repetitive structure (Eid et al., 2009; Booher et al., 2015; Wilkins et al., 2015; Grau et al., 2016; Peng et al., 2016; Quibod et al., 2016; Tran et al., 2018). Collectively, TALE repertoires (TALomes) encoding polymorphic groups that have contrasted abilities to induce susceptibility target genes potentially underlie host adaptation at a small evolutionary scale (Doucoure et al., 2018). The *Xo* group has clearly undergone significant evolution influenced by geography, environment, and host, and TALomes can provide critical insight into how these events occur.

*Xanthomonas campesiris* pv. *leersiae* (*Xcl*), a pathogen of *L. hexandra*, was previously shown to group distinctly from *Xo* by host range and phylogenetic analysis (Fang et al., 1957; Parkinson et al., 2009). However, using a multi-locus sequence alignment (MLSA) analysis Triplett et al. (2011) showed that *Xcl* strain NCPPB4346, which was isolated from southern cutgrass in China, groups within the *Xo* cluster, yet it could not be assigned to any described pathovar. A more recently isolated strain, BA123 from weeds in Burkina Faso, showed high sequence similarity with *Xcl* NCPPB4346 based on a MLSA analysis as well as the presence of TALEs (Wonni et al., 2014). Together, the two strains form a distinct genetic cluster within *X. oryzae*.

Prediction algorithms based on the TALE’s specific RVD pattern and their corresponding degenerate DNA code has facilitated identification of plant target genes whose promoters contain EBEs for TALE binding (Doyle et al., 2012; Grau et al., 2013, 2016; Perez-Quintero et al., 2013; Booher and Bogdanove, 2014; Yang et al., 2014). Many S genes are transporters.

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1 www.isppweb.org/about_tppb_naming.asp
(sugar or sulfate) or transcription factors and upon induction facilitate bacterial colonization and symptom development (Hutin et al., 2015; Tran et al., 2018). Although a large body of work is available on TALEs from Xoo and Xoc, no information has been reported on the TALEs from Xcl, how they compare to those in other X. oryzae, and the nature of their predicted targets within Leersia spp. In this study, we used comparative genomics, identification of T3 effectors, TALomes, and disease phenotyping to characterize Xcl. We used gene target prediction algorithms to identify potential Xcl TALE gene targets in draft Leersia genome sequences. Finally, we provide evidence to support renaming Xcl to X. oryzae pv. leersiae (Fang et al., 1957) and will refer to this organism as Xol throughout this work.

**MATERIALS AND METHODS**

**Bacterial Strains and Plant Varieties**

Bacterial strains included in this study are listed in Table 1. Bacteria were cultured on peptone sucrose agar (PSA) at 28°C for plant inoculations. Genomic DNA for sequencing was isolated from Xol strains BAI23, BB 151-3, BB 156-2, NCPPB4346, and NJ 6.1.1 grown for 48 h on nutrient agar at 28°C (Lang et al., 2014; Wonnii et al., 2014). Barley (Hordeum vulgare L. cultivar Morex), wheat (Triticum aestivum cv. Chinese Spring) and tobacco (Nicotiana tabacum) were grown in a growth chamber at 22°C, 50% relative humidity, and 16 h of light. Rice (Oryza sativa) varieties included in pathogenicity assays were Azucena, Carolina Gold, Cypress, IR64, and Nipponbare.

Southern cutgrass (L. hexandra) was collected in Texas, United States, and seed was propagated at Colorado State University. The seed was scarified then germinated in porous ceramic silica (Greens Grade, Profile Products, LLC, Buffalo Grove, IL, United States) and 0.5x Hoagland’s solution with the following modifications: 2.5 mM KNO₃, 1 mM MgSO₄, 3 mM KH₂PO₄, 2.5 Ca(NO₃)₂, 0.05 mM FeSO₄, and 0.1 mM (Na₂)₂EDTA (Hoagland and Arnon, 1950). Seed was incubated in a petri dish in the dark for 8 days, then in the light for 30 days at 28°C. Germinated seeds were transplanted into 1-gallon pots with equal parts Greens Grade and ProMix BX (ProMix, Quakertown, PA, United States), and grown in a greenhouse (27 ± 1°C, 16 h day length, and 80–85% relative humidity). Additional plants were obtained from the two mother plants via rhizome propagation. Propagation began 30 days after planting with subsequent propagation every week to allow time for new growth. To promote root growth, plants were placed in the dark for 24 h.

**Pathogenicity Assays**

Rice varieties were inoculated at 4–5 week-old with suspensions (10⁸ CFU mL⁻¹) of bacterial strains listed in Table 1. Bacterial suspensions were both infiltrated into the intercellular spaces of rice leaves on either side of the main vein with a needleless syringe and inoculated by leaf clipping as described (Kauffman et al., 1973; Reimers and Leach, 1991). Two leaves were inoculated on each of three to six separate plants; water was included as a negative control. The entire experiment was conducted twice. Lesions were measured at 12 days post inoculation (dpi), and bacterial numbers in planta were quantified as previously described (Verdier et al., 2012).

**Molecular Diagnostics**

To test relationships of Xol to Xo (Notomi et al., 2000; Triplett et al., 2011; Wonnii et al., 2014), a diagnostic multiplex and loop mediated isothermal amplification (LAMP) PCR were used (Lang et al., 2010, 2014; Wonnii et al., 2014). Previously described universal US Xo primers were also tested to differentiate Xol from this novel US clade within the species (Triplett et al., 2011). UniQ Primer was employed to compare draft Xol genomes (BAI23 and NCPPB4346) and generate primers specific to Xol as previously described (Ash et al., 2014; Lang et al., 2014, 2017; Juanillas et al., 2018). Specificity was validated by screening Xol primers against diverse pools of bacterial genomic DNA (Table 1). Primers, expected product size, and optimal annealing temperatures are listed in Supplementary Table S1.

**Genome Sequencing and Assembly**

Long read, SMRT sequencing (PacBio, Menlo Park, CA, United States) data were generated for five Xol strains and for the Xo strain X11-5A, to be used as an outgroup. DNA for SMRT sequencing was isolated by culturing strains on nutrient agar for 48 h then using the Genomic DNA buffer set and Genomic-tips according manufacturer instructions (Qiagen, Valencia, CA, United States). SMRT sequence was assembled using HGAP v4 (PacBio, Menlo Park, CA, United States). Genomes were circularized using circulator (Hunt et al., 2015). Assemblies and raw data have been deposited in NCBI (BioProject IDs PRJNA522807 and PRJNA522811; BioSample accessions SAMN03862116, SAMN02469650, SAMN10956066-68, SAMN10956070; raw sequencing files SRXS417793-98; Assembly accessions CP036251-56). Assembly CP036251 (X11-5A) replaces draft assembly GCF000212755.1 and assembly CP036253 (NCPPB4346) replaces draft assembly GFC001276975.1 (also in Bioproject PRJNA257008). Accessions for all genomes used in this study are listed in Supplementary Table S2.

**Phylogenomics and Bioinformatic Analyses**

In addition to the five Xol sequenced strains, all completely sequenced X. oryzae genomes were obtained from the NCBI to be used for comparisons, as well as representative genomes from other Xanthomonas species. All genomes and accessions can be found in Supplementary Table S2.

Average nucleotide identity (ANI) values were obtained using the ANI-matrix script from the enveomics collection (v1.3) (Rodriguez-R and Konstantinidis, 2016). Parsimony trees based on pan-genome SNPs were obtained using KSNP (v3.0) (Gardner et al., 2015). Multi-locus sequence analysis was made by identifying 33 housekeeping genes in all genome using Amphora2 (Wu and Scott, 2012). Concatenated amino acid sequences of these genes were then aligned using MUSCLE (v3.8.31) (Edgar, 2004), and neighbor-joining...
TABLE 1 | Bacterial strains used in phenotyping and molecular diagnostics.

| Species                      | Strain | Origin          | Host                  | Reference or source                  | Xol5 | Xol7 |
|------------------------------|--------|-----------------|-----------------------|--------------------------------------|------|------|
| Burkholderia andropogonis   | 3549   | Zea mays        | Oryza sativa          | L.E. Claflin                         | –    | –    |
| Escherichia coli             | DH5a   |                 |                       |                                      | –    | –    |
| Pseudomonas fuscovaginiae    | SE-1   | Philippines     | Oryza sativa          | G. Ash                               | –    | –    |
| P. syringae pv. syringae     | M108   | United States   | Solanum lycopersicum  | H.F. Schwartz                        | –    | –    |
| Xanthomonas sp.              | M136   | Mali            | O. sativa             | V. Verdi                            | –    | –    |
| Xanthomonas sp.              | SHU100 | Philippines     | O. sativa seed        | C.M. Vera Cruz                       | –    | –    |
| X. campestris pv. alfalfiae  | KX-1   | United States   |                       | L.E. Claflin                         | –    | –    |
| X. c. pv. campestris         | X1910  | United States   | Brassica oleracea     | N. Dunlop                           | –    | –    |
| X. c. pv. pelargonii         | X5     | United States   | Geranium sp.          | L.E. Claflin                         | –    | –    |
| X. euvesicatoria             | 8S-10  | United States   | Capsicum frutescens   | A. Bogdanove                         | –    | –    |
| X. euvesicatoria             | O177   | United States   | Allium cepa           | H.F. Schwartz                        | –    | –    |
| X. oryzae                    | X11-5A | United States   | O. sativa             | Triplett et al., 2011                | –    | –    |
| X. o. pv. leersiae           | BA23a  | Burkina Faso    | Weeds                 | V. Verdi, Wonni et al., 2014         | +    | +    |
| X. o. pv. leersiae           | BB 151-3 | Uganda       | O. sativa             | B. Yang, R. Oliva, G. Onaga          | +    | +    |
| X. o. pv. leersiae           | BB 156-2 | Uganda       | O. sativa             | B. Yang, R. Oliva, G. Onaga          | +    | +    |
| X. o. pv. leersiae           | NCPPB4346a | China     | Leersia hexandra      | Triplett et al., 2011; Wonni et al., 2014 | +    | +    |
| X. o. pv. leersiae           | NJ 6.1.1 | Mali           | O. sativa             | This study                           | +    | +    |
| X. o. pv. oryzae             | A3857  | India           | O. sativa             | J.E. Leach                           | –    | –    |
| X. o. pv. oryzae             | BA3a   | Burkina Faso    | O. sativa             | V. Verdi                            | –    | –    |
| X. o. pv. oryzae             | MA11   | Mali            | O. sativa             | V. Verdi                            | –    | –    |
| X. o. pv. oryzae             | NA8    | Niger           | O. sativa             | V. Verdi                            | –    | –    |
| X. o. pv. oryzae             | PX066  | Philippines     | O. sativa             | C.M. Vera Cruz                       | –    | –    |
| X. o. pv. oryzae             | PXO99a | Philippines     | O. sativa             | J.E. Leach                           | –    | –    |
| X. o. pv. oryzae             | Xoo4   | Thailand        | O. sativa             | J.E. Leach                           | –    | –    |
| X. o. pv. oryzae             | Xoo199 | Korea           | O. sativa             | S.H. Choi                           | –    | –    |
| X. o. pv. oryzae             | BL583  | Philippines     | O. sativa             | C.M. Vera Cruz                       | –    | –    |
| X. o. pv. oryzae             | BL5105 | Philippines     | O. sativa             | C.M. Vera Cruz                       | –    | –    |
| X. o. pv. oryzae             | BLS256a | Philippines     | O. sativa             | A. Bogdanove                         | –    | –    |
| X. o. pv. oryzae             | BLS305 | Philippines     | O. sativa             | C.M. Vera Cruz                       | –    | –    |
| X. o. pv. oryzae             | MA14   | Mali            | O. sativa             | V. Verdi                            | –    | –    |
| X. o. pv. oryzae             | MA10a  | Mali            | O. sativa             | V. Verdi                            | –    | –    |
| X. translucens               | LH2-1  | United States   | L. hexandra           | This study                           | –    | –    |
| X. t. pv. cerealis           | NCPPB1944 | United States | Bromus inermis        | V. Verdi                            | –    | –    |
| X. t. pv. phaseolatensis     | PDDCC5744 | United States | Phleum pretense      | C. Stevens                           | –    | –    |
| X. t. pv. translucens        | B76    | United States   | Hordeum vulgare       | N. Tisserat                          | –    | –    |
| X. t. pv. translucens        | NCPPB2389 | India         | H. vulgare            | C. Braggard                         | –    | –    |
| X. t. pv. translucens        | UPB787 | Paraguay        | H. vulgare            | C. Braggard                         | –    | –    |
| X. t. pv. undulosa           | UPB513 | Mexico          | T. aestivum           | C. Braggard                         | –    | –    |

*aStrains tested for pathogenicity to Oryza sativa and Leersia hexandra.*

Automated annotation of proteins used in this manuscript for all genomes was made using Prokka (v1.14-dev) (Seemann, 2014), annotation for public versions available in the NCBI was made with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Groups of orthologs and trees based on presence/absence of ortholog genes were generated using OrthoFinder (v 2.2.6) (Emms and Kelly, 2015). Dotplots of whole genome alignments were obtained using Gepard (v 1.4, using word length 100) (Krum siek et al., 2007). Genome duplications were then quantified using minimap (v.1) (Li, 2016) as implemented in minidot2 (parameters = –g 1000 –k 50

2https://github.com/thackl/minidot
Colinear gene regions were identified using DAGchainer (Haas et al., 2004) (parameters = −E 1e−20 −s −g 2000 −x 200 −A 4) using the blast results from orthofinder as inputs. Insertion sequences (ISs) were identified using ISEScan (v1.6) (Xie and Tang, 2017).

Non-TALE T3 effectors were identified by BLASTP (v. 2.6.0+) results were filtered keeping hits with −e value < 0.0001, > 0% identity in >40% the query length) (Boratyn et al., 2013) of consensus effectors sequences obtained from http://xanthomonas.org/ against the protein sequences obtained using Prokka. TALE sequences were extracted from each genome using in-house Perl scripts³. Neighbor joining trees were generated from concatenated nucleotide TALE N and C terminal sequences, alignments were made using MUSCLE (Edgar, 2004) and trees were generated using functions (dist.ml; model = “JC”, NJ, bootstrap.pml) of the phangorn (v2.4.0) (Schliep, 2011).

Transcription activator-like effector repeat sequences were aligned using DisTAL v1.2 (Pérez-Quintero et al., 2015) and neighbor joining trees were generated based on DisTAL genetic distances using the R package ape (Paradis et al., 2004). TALE groups were defined using the function cutree in R (height = 4.8) on the DisTAL tree. Predictions for TALE binding sites were made using Talvez on the promoters (−1 kb upstream of the translation start site) of annotated genes in the L. perrieri (v1.4) and O. sativa cv. Nipponbare (v. MSU7) as previously described (Pérez-Quintero et al., 2013).

RESULTS

Xol Is Pathogenic to Rice and Southern Cutgrass

To better understand the biology of Xol, we established a host range by screening for pathogenicity on rice and southern cutgrass (L. hexandra) using different inoculation techniques. Relative to the virulent Philippine Xoc strain BLS256, the Xol strains were less aggressive to rice, and caused less expansive water-soaked leaf streaking on several rice varieties. Xol BA123 was more aggressive than NCPPB4346 on rice varieties Cypress and IR64, and caused more disease than the US Xo strain X11-5A on Azucena. Rice variety Carolina Gold was resistant to Xol, exhibiting a hypersensitive response and no lesion expansion after infiltration. Both Xol strains caused longer lesions on southern cutgrass than Xo, but were not as aggressive as Xoc BLS256 on rice. Xo produced water-soaked spots at the point of infiltration that did not expand on southern cutgrass (Figure 1).

After clip inoculations, lesions caused by Xol did not expand on rice or southern cutgrass, unlike those caused by the vascular pathogen Xoo (Supplementary Figure S1). When infiltrated into leaves, populations of Xol were equivalent to Xoc BLS256 and Xo X11-5A on rice cvs. Nipponbare or Azucena after 72 hpi (Figure 2). On southern cutgrass, their native host, Xol grew to a significantly higher population than Xo X11-5A. Xol did not cause disease on wheat or barley (Supplementary Figure S2). Phenotyping Nicotiana species can serve as a screen for the ability of microbes, particularly Xanthomonas spp., to elicit a non-host resistance response (Gonzalez et al., 2007). Xol caused minor chlorosis, but not water soaking nor a hypersensitive response, when infiltrated into N. tabacum, similar to the phenotype caused by Xoo PXO99A and Xoc BLS256 while Xoo BA13 and Xo US 11-5A caused a strong hypersensitive response at the site of infiltration on N. tabacum similar to prior reports (Gonzalez et al., 2007) (Supplementary Figure S2).

Our studies show that Xol is pathogenic to southern cutgrass and mildly pathogenic on diverse varieties of rice, but does not cause disease on barley or wheat, and that some rice varieties exhibit resistance to Xol. After inoculation, Xol causes symptoms on rice that are most similar to Xoc, i.e., expanding lesions when introduced into the intercellular spaces (leaf infiltration) and no spreading lesions when introduced into the xylem vessels (clipping).

X. campestris pv. leersiae Belongs to the X. oryzae Species and Is Phylogenetically Close to Xoc

Amplification of Xol DNA with primers specific for Xo but not Xoc, Xoo, or US Xo suggested that Xol were related to X. oryzae, but distinct from other Xo pathovars (Lang et al., 2010; Triplet et al., 2011) (Supplementary Figure S3). We then used SMRT sequencing technology to derive complete genomes of five available Xol strains from China (NCPPB4346), Burkina Faso (BAI23), Mali (NJ611), and Uganda (BB151-3 and BB156-2). We calculated pairwise ANI among these and all fully sequenced X. oryzae genomes (Rodriguez-R and Konstantinidis, 2016). This analysis showed that Xol strains were 99–100% identical to one another, and were 97–99% identical to US Xo, Xoc, and Xoo. Xol were most similar to Xoc (~98.5%). They were 76–91% similar to other Xanthomonas species. X. vasicipa was the next most similar Xanthomonas species to oryzae, sharing 91% ANI with Xol and all Xo (Figure 3). We generated parsimony phylogenetic trees based on pan-gene SNP using kSNP3 (Gardner et al., 2015), which showed again that Xol strains were closely related to Xoc (Figure 3). Neighbor-joining trees generated based on MLSA and on presence/absence of ortholog families showed similar groupings, although the placement of African Xoo in the tree was variable (Figure 3 and Supplementary Figure S4).

These combined data indicate that our sequenced strains are more closely related to Xo than other Xanthomonas species and therefore, combined with previously reported MLSA data (Triplet et al., 2011; Wonnii et al., 2014), we recommend that the formal taxonomic placement of Xol be included in the species “oryzae.” Further biological support for this shift from host range and effector repertoires is described below.

To avoid misidentification of Xol as a Xoc or Xoo in future studies, genomes were compared to identify regions of specificity to base diagnostic primer design with UniqPrimer (Juanillas et al., 2018). Two primer sets were validated for specificity against over 30 closely and distantly related bacteria (Supplementary Table S1). Both primer sets consistently amplified only control Xol strains and did not amplify any other bacterial strain tested (Table 1).

³https://github.com/alperrezq/experimentTAL.
FIGURE 1 | X. oryzae (campestris) pv. leersiae (Xcl) and X. oryzae cause water soaking on reciprocal hosts, but virulence is highest on original host. Phenotypes and quantitative lesion lengths caused by Xanthomonas oryzae (Xo) X11-5A, X. o. pv. oryzicola (Xoc) BLS256 and X. o. pv. leersiae NCPPB4346 on (A,C) diverse rice varieties and (B,C) wild Leersia hexandra were measured 12 days post infiltration inoculation. An asterisk denotes a significant difference between strains on each variety (p ≤ 0.05). Error bars represent ± SD.

Rice Associated X. oryzae Show High Genome Plasticity Compared to X. oryzae pv. leersiae

We generated dotplots to visualize pairwise whole genome alignments to further compare the genomes of X. oryzae strains. These alignments showed several genome rearrangements between the different Xol strains with respect to each other and to other X. oryzae groups. Curiously, we noticed that self-alignments of genomes of pathovars oryzae and oryzicola overall showed more genomic duplication than those of Xol (Figure 4A). To quantify this, we identified colinear regions within each genome that showed these possible genomic duplication events are present in different frequencies in each X. oryzae lineage; and that they are more frequent in Xoc and Asian Xoo, and less frequent in Xol and African Xoo (Figure 4B). Notably, all X. oryzae examined exhibited overall more genomic duplications than other Xanthomonas species (Figure 4B).

We also identified colinear arrangements of homologous genes within each genome, meaning genome duplications that involve multiple genes, and again, these were more frequent in Xoc and Xoo than in Xol (Figure 4C). Since some of these duplications may be mediated by duplicative transposition we annotated and quantified insertion sequences (ISs) in the different X. oryzae genomes, which indeed revealed a higher frequency of IS in the groups with higher amounts of duplicated regions (Figure 4). The distribution of IS families was overall similar among X. oryzae, with Xol’s most resembling the distribution of African Xoo (Figure 4E).

Non-TAL T3E Repertoires of Xol Are Similar to Xoc

Computational prediction of T3E among annotated proteins in each genome revealed that the Xol T3E repertoire resembles more closely that of Xoc. Nonetheless, some features are unique to Xol, specifically all Xol strains possess a xopD gene that is absent in all other Xo genomes. XopAH is also present in all Xol strains and is shared only by two Xoc strains. On the other hand, Xol strains lack xopO which is present in all Xoc strains, and some Xol strains seem to lack the otherwise universal effectors xopW and xopK (Supplementary Figure S5).

Xol Strains Have Distinct TALE Repertoires With Some Similarities to African Xoo

Previous Southern blot analyses using conserved TALE probes predicted that Xol BAI23 and NCPPB4346 had five and four
**FIGURE 2** | *X.* oryzae pv. leersiae can grow and colonize rice as effectively as rice pathogens and rice pathogens can colonize *Leersia hexandra* as effectively as *X.* oryzae pv. leersiae. Bacterial population growth in leaves of rice varieties Nipponbare and Azucena and *L.* hexandra inoculated with *X.* oryzae X11-5A, *X.* oryzae pv. oryzicola BLS256, and *X.* oryzae pv. leersiae NCPPB4346 were quantified at 0 and 72 h post inoculation (hpi). Population sizes were measured in a 5 cm leaf segment infiltrated with each strain. Error bars represent ± SD of six independent leaves, and letters denote treatments significantly different from one another on each variety (*p* ≤ 0.05).

**FIGURE 3** | *X.* oryzae pv. leersiae (Xol) is a member of *X.* oryzae (Xo) and is closely related to *X.* oryzae pv. oryzicola (Xoc). The heatmap shows pairwise average nucleotide identity (ANI) values between fully sequenced *X.* oryzae genomes. (Left) Consensus parsimony tree generated with based on shared pangenome SNPs, numbers in gray indicate node support as outputed by kSNP3, heatmap rows are ordered according to this tree. (Top) MLSA neighbor-joining tree based on concatenated alignments of 33 housekeeping genes, numbers in gray indicate bootstrap support for branches, and heatmap columns are ordered according to this tree. *X.* oryzae pv. oryzae is abbreviated as Xoo and *X.* vasicola pv. vasculorum as Xvv. All species abbreviations are followed by strain name.
TALEs, respectively (Wonni et al., 2014). However, Southern blot analysis cannot resolve TALEs that are close in size. We found in our whole genome sequences that Xol strains contained 12 or 13 TALEs each, which is more than the nine TALEs per genome found in African Xoo, and less than what is commonly found in Xoc (22–29 TALEs) and Asian Xoo (13–20 TALEs). No truncated TALEs were identified in any Xol genome. As previously reported, no TAL effectors were found in the Xo strain X11-5A (Ryba-White et al., 1995; Triplett et al., 2011).

Phylogenetic trees based on the N and C termini of X. oryzae TALEs showed that Xol TALEs form a distinct group, but seem to be close to African Xoo TALEs, despite the overall genomic similarities with Xoc (Supplementary Figure S6). We also constructed trees based on similarities in the CRR of using DisTAL, which grouped Xol and African Xoo together in a subgroup that also includes Xoc (Figure 5A). We used the DisTAL tree to define TALE groups based on repeat region similarities, Xol TALEs were classified in 12 groups (Figure 5B).
One of the groups contained TALEs from Xoc and Xol strains, while all the others were exclusive to Xol. Of these, seven groups were present in all five Xol strains. One of these groups, present in the four African Xol strains, contains the RVD combination “TI” which has not been previously reported in other species.

All Xol strains contained at least two TALEs that were classified within the same group, that is, within each Xol genome there are at least two TALEs with nearly identical repeat arrangements (Figure 5B). By examining pairwise genetic distances between the repeat regions of TALEs, we saw that TALEs from Xol are on average more similar to each other than TALEs within other X. oryzae groups, indicating that they are possibly less diversified and/or more redundant (Figure 5C).

**Xol TALE Targets in Cutgrass and Rice Are Different From Xoc and Xoo Targets**

Talvez (Pérez-Quintero et al., 2013) was used to predict host targets for each TALE in our dataset in the promoters of annotated genes in the L. perrieri (v1.4) and O. sativa (vMSU7) genomes. We identified ortholog pairs between both genomes using reciprocal BLAST. Comparisons of the predictions between both genomes for Xol TALEs revealed very few cases where an ortholog pair was predicted to be a target in both genomes (Supplementary Figure S7), and highlighted that the promoters (and thus the targeted genes) in these two hosts are very different.

We then compared predictions for all Xol lineages in both genomes and calculated the overlap between predictions for each
strain. As a result, we saw that each X. oryzae group has a distinct group of predicted targets with relatively little overlap with other groups. In the case of Xol, the highest overlap was found with predictions for Xoc TALEs (10–11% shared predicted targets) (Figure 6). Given the differences in the genomes of their hosts and that Xol TALEs have unique repeat sequences, we expect their targets to be likewise unique. Additionally, we looked for orthologs of known susceptibility (S) genes targeted by Xoc or Xoo TALEs (e.g., SWEETs, OsSULTR3;6) and queried whether they were among the top predictions for Xol TALEs. No known Xoc or Xoo target homolog was found. It is, however, possible, that while not targeting the direct orthologs of these S genes, Xol TALEs may be inducing similar functions, since the predicted targets contain genes annotated with similar functions to known S genes including sulfate transporters, nodulins and various families of transcription factors (Supplementary Table S3). Expression data and further experiments are necessary to effectively identify the biological mechanisms of these TALEs.

**DISCUSSION**

*Xanthomonas oryzae* pv. *leersiae*, which has been isolated from the pervasive weed species *L. hexandra* surrounding rice paddies
Xanthomonas oryzae pv. leesiae colonize and cause water-soaked lesions on rice leaves, but the southern cutgrass isolates are not as aggressive as Xo isolated from rice. The lesions caused by Xol were phenotypically similar to rice BLS caused by Xoc and these strains did not cause disease when introduced into rice or southern cutgrass by leaf-clipping. Thus, we suggest that Xol are not systemic pathogens, and are more like Xoc than the systemic relative Xoo.

T3Es, as important contributors to bacterial pathogenicity, may define host range, and can also inform lineages and evolutionary relationships among different populations of related bacteria (Arlat et al., 1991; Hajri et al., 2012; Wonni et al., 2014; Schwartz et al., 2015). Studies in X. oryzae have shown that different lineages are shaped by T3E repertoires and reflect phenotypic adaptation to their agroecosystems (Hajri et al., 2012; Quibod et al., 2016). Likewise, this study sought to uncover similarities between Xo and Xol effector repertoires that could further inform their evolutionary lineage. Out of a set of previously defined core effectors for X. oryzae [avrBs2, avrBs3 (TALEs), xopL, xopN, xopP, xopQ, xopV, xopW, xopY, xopAA, xopAB, xopAE, and xopF1] (Hajri et al., 2012), Xol strains contain all except xopW, which is absent in three of the five strains. Although present in strains BAI23 and NJ611, xopW contains a large IS. This IS most likely prevented its amplification in previous studies (Wonni et al., 2014). Interestingly, this same IS was identified in other African Xoc strains, consistent with a shared evolutionary origin with Xol (Hajri et al., 2012). On the other hand, xopD is present in Xol but absent in other X. oryzae. XopD is a SUMO protease mimic that suppresses host defense responses during Xanthomonas euvesicatoria infection (Kim et al., 2008, 2013). In addition to Xol, xopD is predicted to be present in X. campestris pv. campestris, X. euvesicatoria, and Acidovorax citrulli. Absence in other Xo suggests an independent acquisition by Xol. Conversely, loss of this effector at some time by Xoo or Xoc could have occurred, but further validation of these hypotheses is necessary. Since it is not known what level of virulence, and/or host specificity any T3E conveys for Xol, future work should include functional validations.

We also assessed TALE diversity in Xol strains, using genomic assemblies based on long reads generated using SMRT sequencing. While according to phenotyping, phylogenomics, and non-TALE Type III effector repertoires, Xol most closely resembles Xoc, the Xol TALE sequences more closely resemble African Xoo. Xol and African Xoo also possess, on average, smaller TALomes (TALEs per genome) than Xoc or Asian Xoo. Furthermore, their TALEs also have less diverse repeat arrangements as evidenced by shorter genetic distances in pairwise TALE repeat alignments. A possible explanation for this feature is that TALEs from Xol and African Xoo more closely resemble the ancestral TALE repertoire for the group and have not undergone the extensive diversification found in Xoc and Asian Xoo. A hypovirulent strain of Xoc was isolated from rice in the Yunnan province of China that also contains nine TALEs. This strain was used in heterologous expression assays to determine targets and function of Tal7 (Cai et al., 2017). Unfortunately, the TALome of this strain is unavailable at this time and it is unclear if this strain is related to the Xol strains characterized in this study.

The expansion of Xoc and Xoo TALomes is curious since many of the TALEs they carry seem not to be required for virulence and some may even have redundant functions (Pérez-Quintero et al., 2013; Cernadas et al., 2014). Given their repetitive nature and a general tendency toward homogenization of repeats, TALEs have been proposed as being selected for evolvability, that is, selected for their ability to quickly recombine (Schandry et al., 2016). Having an expanded TALome would further allow frequent recombination and generation of new TALE variants, as reflected in bigger evolutionary distances between repeat arrangements. This selection of a bigger TALome has been proposed to be driven by extensive breeding for resistance in the host plant (Schandry et al., 2018). When exposed to a resistant host population, a pathogen population can benefit from carrying a heterogeneous and redundant set of effectors, since preexisting isolates harboring a set advantageous for the new conditions would then be selected, in what has been proposed as a type of evolutionary “bet-hedging” strategy (Win et al., 2012). It is then possible that Xoc and Asian Xoo have historically encountered more resistance, possibly related to domestication of its primary host, than Xol and African Xoo, leading to a selection for expanded TALomes.

At least two resistance genes (Xa1 and Xa10) in O. sativa specifically recognize TALEs (Ji et al., 2016; Triplet et al., 2016), and Xoc and Xoo seem to have benefited from their expanded TALomes by selecting TALE variants (ITALES or truncTALES) that can specifically suppress resistance mediated by these genes (Ji et al., 2016; Read et al., 2016). Meanwhile Xol infecting L. hexandra, as African Xoo originally infecting Oryza glaberrima (Gonzalez et al., 2007), may have never encountered similar resistance in its host, and thus lacks these TALE variants and cannot cause disease on varieties carrying Xol (Figure 1).

TALome expansion may have been accompanied by, or be a consequence of, higher genome plasticity in the X. oryzae clade as evidenced by a high amount of genome and gene duplication and a high frequency of IS elements, these measures being generally lower in Xol. Within the clade, differences

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may be once more related to resistance in the host, with the more plastic genomes (Asian *Xoo*) matching more variable host populations. The requirement of plastic genomes in the clade as a whole has been hypothesized to be a consequence of rice cultivation through millennia (Salzberg et al., 2008; Bogdanove and Voytas, 2011), and suggest the ancestor of the clade faced an already variable population. Which then raises the question of where *Xol* falls within this scenario of adaptation to a cultivated crop?

Rice and southern cutgrass are closely related members of the Poaceae. *Leersia* species are often used as an outgroup in phylogenetic and, most recently, genomic investigations (Copetti et al., 2015). Evidence of genome duplication events and defense response genes shared by rice and *Leersia* species has been reported (Jacquemin et al., 2009; Xiao et al., 2009). It is plausible that based on their genetic and evolutionary relatedness that respective pathogens of *Oryza* and *Leersia* evolved independently. The US strains of *Xo*, which do not contain intact TALEs, could have been progenitors of other *Xo* pathogens, having acquired TALEs over time to enhance virulence on rice or *Leersia* spp. (Tripplet et al., 2011). Significant trade between the United States, Africa, and Asia could allow for movement of strains across continents. However, it is not clear if *Xol* is currently present in the United States, despite early reports of *L. hexandra* being an alternate host for *Xoo* (Gonzalez et al., 1991). Alternatively, this system may represent a sympatric scenario where *Xoo*, *Xoc*, *Xo*, and *Xol* all lived in the same habitat, providing the opportunity to exchange genetic material and to adapt to their respective host while maintaining basic homology (Jacques et al., 2016). In this scenario, given its similar infection biology and overall similarities to *Xoc*, *Xol* may be a specialized subgroup originating from a *Xoc*-like population able to colonize southern cutgrass, or vice versa.

How specifically adapted *Xol* strains are to either *L. hexandra* or rice remains a fascinating question, since *Xol* could potentially represent an emerging pathogen for rice. Here we have shown that *Xol* can, to some extent, infect rice, and two of the isolates sequenced in this work were originally recovered from symptomatic rice leaves in a field (BB 151-3 and BB 156-2). To get insights into host adaptation, we attempted to predict targets for *Xol* TALEs in *L. hexandra* using *L. perrieri*, the only available genome from the genus, as proxy and *O. sativa*. Overall, the predictions indicate that *Xol* TALEs induce different sets of genes than other *X. oryzae*, and that different genes are induced in rice and *Leersia* sp. given that relatively few orthologues are predicted to be targets in both genomes. Of particular interest, given the phenotypic similarities with *Xoc*, is the predicted targeting of genes annotated as sulfate transporters. Four genes in the *L. perrieri* genome corresponding to orthologs of sulfate transporters from rice (LOC_Os03g09940, LOC_Os03g09970, LOC_Os03g09980, and LOC_Os09g06499) were predicted to be targeted by TALEs from at least one *Xol* strain with high prediction scores (Supplementary Table S3). The primary virulence target of Tal2g from *Xoc* is OsSULTR3;6 which is a member of the sulfate transporter family 3 (Takahashi et al., 2011; Cernadas et al., 2014). It is feasible that the TALEs targeting sulfate transporters in *Leersia* mirror the virulence function of Tal2g. However, transcriptomic data and further biological validation are required since it is still possible that the few-shared genes in the predictions are true targets and similar functions are required for virulence in both hosts.

**CONCLUSION**

In summary, we propose *Xol*, which was isolated from rice and southern cutgrass (*L. hexandra*), a weedy grass closely related to rice, as a new member of the *X. oryzae* species. Genomic analysis and disease phenotyping on various hosts demonstrated the close relationship of *Xol* to the rice pathogens *Xoo* and *Xoc*. T3E and TALE content of the *Xol* indicated that this group of organisms uses similar virulence mechanisms to the rice pathogens. While weeds such as southern cutgrass are not agronomic crops, they are competitors for resources and potential reservoirs of pathogen inoculum, they are important in management considerations for rice growers. Interfering in any agroecosystem requires comprehensive consideration. Certain *Leersia* spp. are used as banker plants for the critically important rice brown plant hopper (Zheng et al., 2017), therefore integrated management of weeds surrounding rice paddies will require prospecting and balance of all possible pests. The fact that they harbor a pathogen group that can also impact rice emphasizes that, in general, more attention should be focused to the surrounding ecosystem in rice production and more broadly in any crop rotation as a general management strategy. Research contributing toward understanding the *Xol*/rice/southern cutgrass pathosystem will be significant for all rice-producing countries.

**AUTHOR CONTRIBUTIONS**

JML, AP-Q, RK, BS, VV, and JEL conceived and designed experiments. JML, AP-Q, RK, ED, and JJ performed the experiments. SS, HD, IK, RO, OK, and VV collected and provided new *Xol* strains. JML, AP-Q, RK, and JZ analyzed the data. RK, RO, OK, BS, VV, and JEL provided resources and supervision. JML, AP-Q, BS, VV, and JEL developed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00507/full#supplementary-material
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