Development of genome-driven, lifestyle-informed primers for identification of the cereal-infecting pathogens *Xanthomonas translucens* pathovars *undulosa* and *translucens*.

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ABSTRACT

Bacterial leaf streak, blight and black chaff caused by *Xanthomonas translucens* pathovars are major diseases affecting small grains. *Xanthomonas translucens* pv. *translucens* and *X. translucens* pv. *undulosa* are seedborne pathogens that cause similar symptoms on barley, but only *X. translucens* pv. *undulosa* causes bacterial leaf streak of wheat. Recent outbreaks of *X. translucens* have been a concern for wheat and barley growers in the Northern Great Plains and Upper Midwest; however, there are limited diagnostic tools for pathovar differentiation. We developed a multiplex PCR based on whole-genome differences to distinguish *X. translucens* pv. *translucens* and *X. translucens* pv. *undulosa*. We validated the primers across different *Xanthomonas* and non-*Xanthomonas* strains. To our knowledge, these are the first multiplex PCR to distinguish *X. translucens* pv. *translucens* and *X. translucens* pv. *undulosa*. These molecular tools will support disease management strategies enabling detection and pathovar incidence analysis of *X. translucens*.

INTRODUCTION

Xanthomonas translucens is a bacterial plant pathogen that limits global cereal and grass production (Sapkota et al. 2020). For cereals, *X. translucens* causes multiple diseases such as bacterial leaf streak (BLS) and bacterial blight (BB) of leaves and black chaff on seeds (Bragard et al. 1997, 1995; Jones et al. 1916; Smith et al. 1919). Reports indicate that *X. translucens* infection can lead to a 40% yield reduction or 8-13% losses in kernel weight (Tubajika et al. 1998; Shane et al. 1987; Forster 1988). In the United States, *X. translucens* is present in several states with ongoing outbreaks across the Upper Midwest region, but the current economic and yield impact of these diseases remain undescribed (Curland et al. 2018; Adhikari et al. 2012; Kandel et al. 2012).

Historically, *X. translucens* taxonomy is based on host specificity, which dictates pathovar nomenclature (Bragard et al. 1995; Curland et al. 2020; Ledman et al. 2021). This host-range based classification system is currently composed of ten described pathovars. The main four cereal-infecting pathovars include *translucens*, *undulosa*, *cerealis* and *secalis* (Table S1). An additional five pathovars infect wild and forage grasses and other monocots (e.g., *graminis*, *arrhenatheri*, *phleipratensis*, *poae*, *phlei*) (Table S1). *Xanthomonas translucens* pv. *pistaciae* was isolated from the dicot pistachio, but recent genetic analysis demonstrated that this pathovar spans multiple genetic groups (Goettelmann et al. 2022; Giblot-Ducray et al. 2009).

Recent efforts in genome sequencing and phylogenomic analysis have allowed the reclassification of *X. translucens* based on evolutionary relationships into three genomic groups I-III (Heiden et al. 2022; Goettelmann et al. 2022; Sapkota et al. 2020). Cereal-infecting pathovars (e.g. *secalis* [Xts], *translucens* [Xtt], and *undulosa* [Xtu]) belong to genomic group Xt-I (Goettelmann et al. 2022). The Xt-I group was also defined using the Life Identification Number (LIN) classification system that provides an average nucleotide identity (ANI)-based coding system to precisely identify genomic subgroups (Heiden et al. 2022; Tian et al. 2020). According to the LINbase classification, all Xt-I group strains are 96% genomically identical. Xtt is the primary pathogenic agent of barley and does not cause disease on wheat but has been sporadically isolated from wheat (Curland et al. 2018). Xtu infects a broad range of hosts (e.g., wheat, barley, and cultivated wild rice) but is most often associated with BLS on wheat (Curland et al. 2021, 2018; Bragard et al. 1997). Xtt colonizes both the host vascular xylem and non-vascular spongy mesophyll tissue; while Xtu remains localized to the non-vascular system (Gluck-Thaler et al. 2020). Phylogenomics demonstrated that host range- and phenotype-based nomenclature often incorrectly categorize pathogens as compared with evolutionary-based genomic analysis, and pathovar typing can frequently misidentify the genetic subgroup type (Goettelmann et al. 2022; Heiden et al. 2022). Pathovar identifications in *X. translucens* most often arose from its host of origin rather than conducting a rigorous host range analysis (Langlois et al. 2017; Vauterin et al. 2000; Rademaker et al. 2006). Xts is considered a separate pathovar because of its isolation from rye, but this *X. translucens* pathovar is genomically nested in the subgroup Xt-I-Xtu (Goettelmann et al. 2022; Reddy et al. 1924). Therefore, based on evolutionary genomics and phenotypic similarity, Xts belongs to the group Xt-I-Xtu.

There is limited to no control of *X. translucens* diseases. As with most bacterial pathogens, antibiotic applications are not effective, and copper-based control is discouraged as many *Xanthomonas* species display tolerance or acquire horizontally-transferred resistance (Lamichhane et al. 2018). Moreover, single-gene plant disease resistance to *X. translucens* diseases is not available in commercial US cereal production. *Xanthomonas translucens* has been identified as a seed-borne pathogen, but it remains unclear which pathovars are seed-transmitted (Duveiller 1990). These multiple barriers to successful management against *X. translucens* pathovars and

recent reports of high disease incidence create an urgent need for resistance discovery (Alizadeh et al. 1994; Curland et al. 2018; Duveiller et al. 1992; Curland et al. 2020; Adhikari et al. 2012). Early diagnosis and surveillance can be used to predict outbreaks, potential yield loss and seed transmission. Currently published methods for *X. translucens* diagnostics include loop-mediated isothermal amplification (LAMP), polymerase chain reaction (PCR), and quantitative PCR (Langlois et al. 2017; Sarkes et al. 2022). PCR diagnostics using the gyrase B gene (*gyrB)* to identify *Xanthomonas* species does not have the specificity to differentiate *X. translucens* pathovars (Parkinson et al. 2009). A LAMP assay developed by Langlois et al. (2017) is the most current method for rapid *X. translucens* group differentiation, but it cannot distinguish between Xtu and Xtt. Additionally, multilocus sequence analysis (MLSA) of four housekeeping genes has been shown to differentiate between Xtt and Xtu, however as MLSA relies on generating and analyzing sequence data it can be time consuming and costlier than PCR detection assays (Curland et al. 2018, Curland et al. 2020, Ledman et al. 2021). As of today, there are no rapid molecular methods publicly available to differentiate Xtt and Xtu.

In this study, we developed a multiplex PCR to detect and differentiate Xtt and Xtu using evolutionary and lifestyle-informed primers (Figure 1). We set a foundation based on whole-genome sequencing-based differentiation, and then used these genomic separations to develop primers unique to Xtu. These gene targets are associated with the pathogenic behaviors (vascular and non-vascular) of these *X. translucens* pathovars. We validated the primer success using pure bacterial cultures covering non-*Xanthomonas*, *Xanthomonas* spp. and *X. translucens* diversity across three independent laboratories. We then tested the primers using bacterial isolates from field samples. Our diagnostic tool improves *X. translucens* pathovar identification using pathogen genetic diversity.

METHODS

Whole genome analysis

To capture the genetic diversity of *X. translucens*, 24 genomes were selected from different *X. translucens* strains. The 24 genomes include 14 strains from Xt-I group, three from Xt-II and seven strains from Xt-III (Table S2). The nucleotide data for each genome was obtained from GenBank. Average nucleotide identity (ANI) was calculated with the tool Genome Matrix from Enveomics (http://enve-omics.ce.gatech.edu/g-matrix/). To obtain their unique Life Identification Number (LIN), genomes were uploaded to LINbase (linbase.org).

Bacterial strains

For primer validation, three bacterial groups were tested: 1) non-*Xanthomonas* plant pathogens, 2) *Xanthomonas* spp., and 3) *X. translucens* pathovars (Table 1, 2). The non-*Xanthomonas* strains were selected because they have been reported as rice or corn pathogens, non-cereal pathogens, or environmental isolates. The *Xanthomonas* spp. strains were selected to cover clade I and II

Xanthomonas including*Xanthomonas* species reported as cereal pathogens or barley commensals (Egorova et al. 2014). The *X. translucens* pathovar strains were selected to cover old and recent Xtu and Xtt isolates.

To test the primers on bacteria from field samples, *X. translucens* was isolated from 10 dried barley leaves donated by producers. The barley leaves were wiped with 70% v/v ethanol and 1 cm^2 of barley leaf tissue was added to 300 µl sterile water. Sample suspensions were incubated at room temperature for 30 minutes. After incubation, 30-50 µl of the liquid was streaked on nutrient agar (NA) plates and incubated for two days at 28°C. Each colony with a *Xanthomonas*-like phenotype was resuspended in 10 µl of sterile water. Five µl of water-colony suspension was blotted on NA, and the remaining 5 µl was added to a PCR tube with 5 µl TE-T (20mM Tris-Cl pH:8, 2mM EDTA pH:8, 0.1%Triton X-100) solution. The colony suspension in TE-T was used for multiplex PCR (*cbsA* and S8.pep primers) as described below. We validated our approach with amplification and sequencing of *gyrB* using previously published PCR primers (Parkinson et al. 2009). If colony suspension was confirmed to be *X. translucens*, 5 µl was added to 500 µl 20-25% sterile glycerol for storage at -80°C.

Primer design

To differentiate among *X. translucens* pathovars, multiplex PCR primers were designed based on *cbsA*, a gene encoding glycosyl hydrolase 6-family cellobiohydrolase. *cbsA* is a gene associated with bacterial adaptation to colonize the plant vascular tissue, and only a fragment remains in wheat-infecting Xtu (Gluck-Thaler et al. 2020). *cbsA* sequence from Xtt UPB 886 (NCBI protein id: MQS43189.1) was used as a BLASTn query to search for all the *X. translucens cbsA* alleles on the NCBI platform. The search included the following parameters: "complete and draft genomes" from "*Xanthomonas translucens* (taxid: 343)" as Dataset and "discontiguous megablast (more dissimilar sequences)" as the BLAST algorithm. The *cbsA* sequences from 24 different *X. translucens* strains were aligned and visualized with NCBI Multiple Sequence Alignment Viewer v1.20.1 using default settings. The phylograms were edited in FigTree v1.4.4 (https://github.com/rambaut/figtree/releases). *cbsA* alleles from six different *X. translucens* pathovars were aligned to manually design two pairs of primers for conventional multiplex PCR. Based on the length and nucleotide diversity of *cbsA*, four primers (*cbsA*-1, *cbsA*-2, *cbsA*-3, *cbsA*-4) were manually designed to amplify the intact *cbsA* in Xtt, Xt-II and Xt-III and the truncated *cbsA* fragment in Xtu.

Xtt and Xtu both amplify a 165 bp *cbsA* fragment and therefore could not be distinguished from a mixed infection. Therefore, a third set of primers was designed based on unique Xtu genes. Seven Xtt strains (UPB 458, UPB 886, UPB 787, XtKm34, CIX95, CIX43, CFBP 2054) and seven Xtu strains (LW16, XtKm12, ICMP11055, P3, 4699, UPB 513, CFBP 2539) were selected to identify unique genes. The 14 complete genomes were annotated with Prokka v1.14.5 (Seemann 2014). The software Roary (v3.13.0) was used to compare the predicted proteins of all genomes and

identify unique Xtu coding genes (Page et al. 2015). The software Mauve (v2015) was used to manually validate Roary results (Darling et al. 2004). From the list of validated unique genes, primers were designed using PrimerBLAST (Ye et al. 2012). Primer Pair Specificity Checking Parameters were changed to have non-redundant database (nr) and "*Xanthomonas translucens*" as organism. The parameters were modified to exclude other *X. translucens* genomes on NCBI.

Polymerase chain reaction amplifications

Bacterial colonies were used as a template for PCR assays using a method adapted from Cormican et al. (1995). Briefly, a 1 µl loopful of each bacterial colony was taken from a two-day old culture grown on NA. The colonies were resuspended in 100 µl sterile water. Twenty µl of the bacterial suspension was mixed with 20 µl TE-T solution and incubated for 10 min at 95°C. The bacterial suspension was vortexed and 2 µl was used as the template in a 25 µl PCR.

Multiplex PCRs with the three primer pairs were performed in a 25 µl reaction containing 0.08 µM each of primers *cbsA*-1, *cbsA*-2, *cbsA*-3, *cbsA*-4, 0.16 µM S8-protease primers, 12.5 µl Quick-Load Taq 2X Master Mix (M0271L, New England Biolabs) and 2 µl template (colony resuspended in TE-T). For the *gyrB* PCR, 25 µl reactions contained 0.08 µM *gyrB* primers (Parkinson et al. 2009), 12.5 µL Quick-Load Taq 2X Master Mix and 2 µl of template. All PCR products were run in 0.7% agarose gels using 100V for 50 min and visualized under blue light with EZ-Vision Bluelight DNA dye 10,000X (VWR). The thermocycling conditions for all PCRs were: i) initial denaturation at 95°C for 30 seconds, ii) 30 cycles at 95°C for 30 seconds, 50°C for 45 seconds, and 68°C for one minute, iii) final extension at 68°C for five minutes, and iv) final temperature 10°C for 10 minutes.

Cross-laboratory validation of *X. translucens* multiplex PCR

To evaluate the multiplex PCR reproducibility, we tested the protocol at three additional laboratories beyond Ohio State including the University of Minnesota, Colorado State University, and the University of British Columbia. Each laboratory followed the PCR protocol and used the reagents described above. All laboratories included water as negative control. The PCR for University of Minnesota included three control strains (Xtc LMG 679, Xtu LMG 892, Xtt LMG 876) and four strains isolated in Minnesota (CIX23, CIX43, CIX86, CIX94). Colorado State University had one control strain (Xtt LMG 876) and two strains isolated from Colorado (Xtt CO 236, Xtu CO 237). The University of British Columbia used two controls (Xtt LMG 876, Xtu LMG 892) and five strains isolated in British Columbia (Xtt-1, Xtt-2, Xtt UBC 026, UBC 028, UBC 029).

RESULTS

Whole genome comparisons

To establish the genomic similarities among *X. translucens* pathovars, we compared whole genomes of 24 different *X. translucens* strains using ANI (Figure 2). As mentioned above, Goettelmann et al. (2022) previously identified three *X. translucens* clades (I, II and III) based on ANI. Our phylogenomic analysis agreed with their findings that Xt-I includes only Xtt and Xtu (Figure 2). As expected, LIN classified these 24 genomes under the same genus and species and genomic groupings (I-III) based on a 95% genome identity threshold. Xtt and Xtu were 97% similar but in distinct LIN subgroups (LIN value I0 and I1, respectively) at the 98% threshold (Figure 2). The limited genomic diversity of the *X. translucens* strains allowed us to design unique primers to differentiate Xtt from Xtu.

Evolutionary genomics- and lifestyle-informed primers

Comparative genomics analysis demonstrated that a single gene, *cbsA,* was present in vascular *Xanthomonas* species, which encodes a GH6-family cellobiohydrolase (Gluck-Thaler et al. 2020). To evaluate *cbsA* gene diversity across *X. translucens* groups, we compared the *cbsA* gene from the three *X. translucens* genomic clades I to III (Figure 3A). To represent the three clades, we used the 24 *X. translucens* genomes used in our genomic comparison analysis (Figure 2) and created a phylogenomic tree based on the BLASTn *cbsA* aligned sequences. The *cbsA* tree displayed a similar organization to the whole-genome comparison tree agreeing with previous findings that *cbsA* is an ancestral *X. translucens* feature (Figure 2, Figure 3A) (Gluck-Thaler et al. 2020).

cbsA alleles alignments revealed that the gene sequences varied in length across genomic groups (Figure 3B). In Xt-I-Xtu, *cbsA* was pseudogenized by a transposon and only a 165 bp fragment remained in the genome (Gluck-Thaler et al. 2020). For Xt-I-Xtt and Xt-III *cbsA* length is 1425 bp and Xt-II *cbsA* is 1394 bp long. The genetic variation in *cbsA* among clades allowed for three different PCR amplicon profiles, using the four *cbsA* primers: profile 1) three bands (165 bp, 600 bp, and 800 bp) for Xtt complete *cbsA*, profile 2) one band (165 bp) for Xtu truncated *cbsA* and profile 3) one band (600 bp) for Xt-II or -III *cbsA.*

Genome-informed primers

The two sets of *cbsA* primers cannot capture a mixed Xtt-Xtu infection. Therefore, to detect mixed infections of the two pathovars or to differentiate their presence in the same field, we designed an additional primer set based on a unique Xtu gene.

Through the comparative genomic analysis of amino acid sequences of Xtt and Xtu we identified 67 unique Xtu genes and selected 13 annotated genes with a known function (not hypothetical genes) to design the primers (Tables 3 and S2). Based on PrimerBlast results, we selected primers for four genes unique to Xtu genomes that were not present in other *X. translucens* pathovars.

Only one primer pair of the four tested (S8.pep-F and S8.pep-R) was Xtu-specific generating a distinct band (Figure S1). This gene target encoded an S8 family peptidase (WP_003469260 from Xtu ICMP 11055 NCBI genome) (Table 3). The role of this gene in Xtu-host interactions remains unknown.

Primer validation

To test our primers and their specificity for Xtt and Xtu, we carried out a colony-based multiplex PCR using *cbsA*-1, *cbsA*-2, *cbsA*-3, *cbsA*-4, S8.pep-F and S8.pep-R. PCR amplification of the three multiplexed primer sets from lysed colonies yielded two band patterns that were distinct between the six Xtt and six Xtu strains tested (Figure 4). For all Xtt strains, three bands were present (800 bp, 600 bp and 165 bp), while the multiplex PCR for all Xtu strains amplified two fragments (450 bp and 165 bp). We also were able to detect a mixed Xtt-Xtu colony PCR with a total of four bands (Figure 4). This PCR primer set also differentiated *X. translucens* genomic groups Xt-II and Xt-III from Xtt and Xtu (Figure 5).

To test the specificity of the primers against non-*Xanthomonas* plant pathogens, we selected five different bacterial species (*Pseudomonas syringae* pv. *glycinea*, *Enterobacter* spp., *Clavibacter nebraskensis, Pseudomonas fuscovaginae* and *Pantoea stewartii*) (Table 1, Figure 5). Two strains (*P. syringae* pv. *glycinea* (PsgR4) and *Enterobacter* spp.) presented some PCR amplification based on the multiplex PCR results, but the band sizes of these organisms did not correlate with the *X. translucens* multiplex PCR profile with bands at 200 bp and 700 bp, respectively. Amplification of five additional *Xanthomonas* species (*X. sacchari, X. hortorum, X. hyacinthi, X. theicola* and *X. prunicola*) (Tables 1 and 2) yielded a band profile that did not match the *X. translucens* multiplex profile (Figure 5).

The multiplex PCR was validated in three different laboratories (University of Minnesota, Colorado State University and University of British Columbia). In all three laboratories, the multiplex PCR separated Xtu from Xtt, with consistent band size profiles for the controls and local and historical isolates (Figure S2).

Bacteria isolated from field samples

The ten barley leaf samples received from growers yielded a total of 17 bacterial colonies with *Xanthomonas*-like morphology. Fifteen of these colonies were identified as a *Xanthomonas* sp. based on the PCR that amplified a fragment of the gene *gyrB*. The *gyrB* PCR product has a single 700bp band for the genus *Xanthomonas*.

The multiplex PCR indicated that 12 of the 15 *Xanthomonas*-positive colonies were *X. translucens* pv. *translucens* (Figure 6B). The colonies C13 and C14 were identified as *Xanthomonas* sp. but not *X. translucens*, therefore the *gyrB* PCR product for both colonies were sequenced. Both fragments had 98.79% identity to *X. hortorum* based on BLASTn search with the nr/nt database.

DISCUSSION

Disease symptoms provide critical information to describe pathogen behavior in agricultural ecosystems. The long history of pathogen typing using pathovar nomenclature provided foundational knowledge to characterize Xtt and Xtu vascular and non-vascular lifestyles and host range (Gluck-Thaler et al. 2020; Curland et al. 2020). This pathovar designation informed previous research that characterized *cbsA* as a vascular pathogen-specific gene, and was the first template for designing a multiplex PCR in this study (Gluck-Thaler et al. 2020). We then used comparative genomics with knowledge about pathogen phenotypes to identify Xtu unique genes and improve our diagnostic primers (Lang et al. 2010).

The *Xanthomonas* genus includes additional species with pathovars that cause respective vascular and non-vascular symptoms on a similar host such as *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* on brassicas; *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* on rice. *cbsA* is present in these vascular *Xanthomonas* spp. but is absent from non-vascular subgroups. As in Xtu, related non-vascular subgroups lack *cbsA* or have a truncated form. Therefore, *cbsA* was used as a prime candidate for differentiating Xtu and Xtt pathovars for diagnostics and could be used as a basis for pathovar differentiation in other related groups of *Xanthomonas* plant pathogens.

In the past, *X. translucens* pathovars primarily were defined by their isolation from or pathogenicity on specific hosts. Currently, whole-genome comparisons are sensitive enough to identify unique traits in genetically close pathogens, such as the Xt-I clade (Goettelmann et al. 2022). Whole-genome analysis will avoid bacterial characterization based on a host of isolation and accurately identify isolates based on comparative phylogenomics.

In this study, we developed a genome-driven and lifestyle-informed pipeline to differentiate relevant cereal pathogens Xtt and Xtu using a multiplex PCR. We tested and confirmed the specificity of the *X. translucens* multiplex PCR primers with non-*Xanthomonas* spp., *Xanthomonas* spp., and *X. translucens* pathovars derived from pure cultures and from bacteria isolated from symptomatic leaf tissue collected from barley fields. From the field samples, we isolated *X. hortorum*, which has been detected in wheat and barley seeds (Egorova et al. 2014). This suggest that barley leaves can be a reservoir for other *Xanthomonas* species and accurate diagnostic tools are necessary to avoid false positives or wrong disease assessment.

To our knowledge, this is the first multiplex PCR that differentiates Xtu and Xtt. We recognize that further analyses are necessary to improve the *X. translucens* multiplex PCR, such as sensitivity, limit of detection and using plant tissue directly from the field. However, we achieved our goal to provide a resource for *X. translucens* pathovar identification, which is currently unavailable. Overall, our pipeline provides a novel, genome-based and lifestyle-informed approach for accurate Xtt and Xtu identification. The multiplex PCR was independently validated by three

laboratories demonstrating that this tool is translatable to other teams and researchers focused on *X. translucens* pathovar identification. This primer set can provide a clear and rapid tool for plant pathogen research and diagnostic labs, which could ultimately lead to improved cereal disease management strategies.

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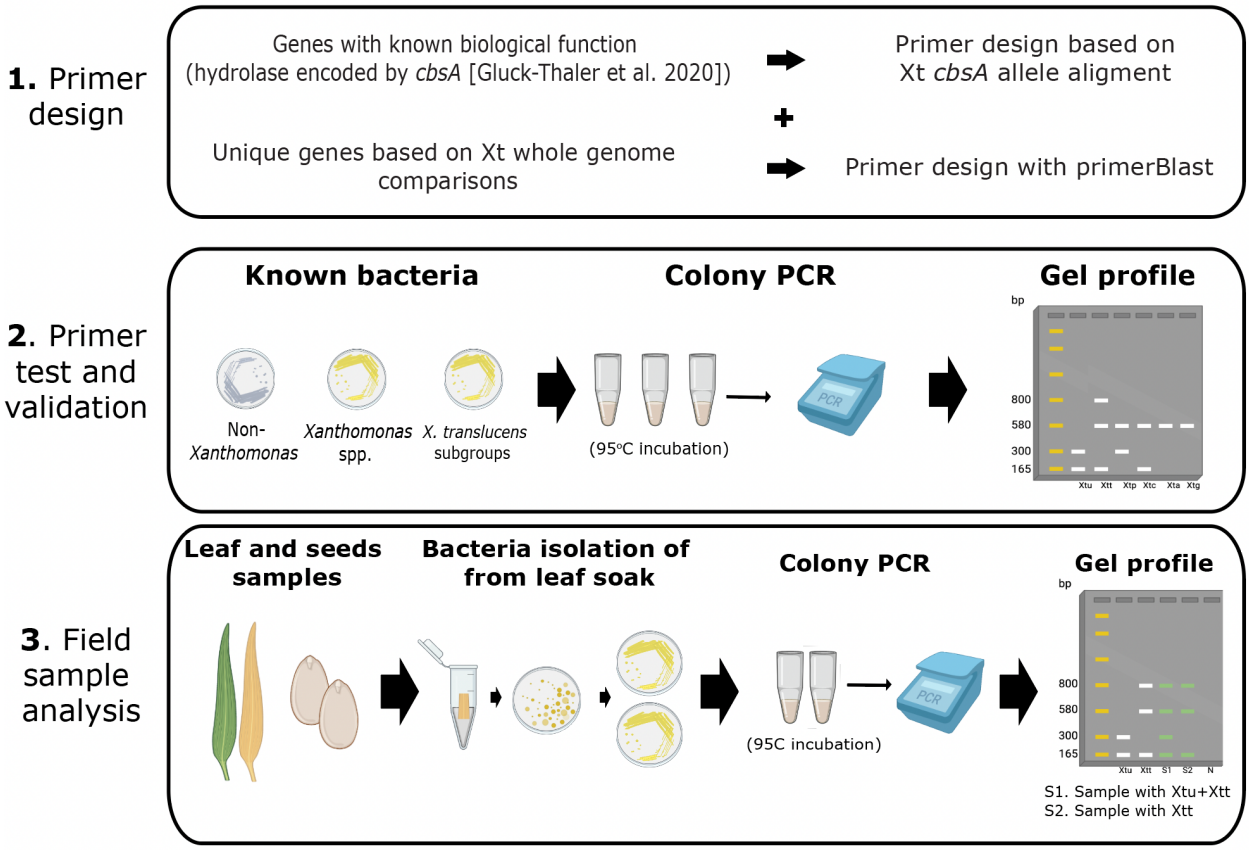

Figure 1. Pipeline for diagnostic primer design using pathogen genomic information. **1)** Primer design. Two approaches were used to design primers – manual and primerBLAST. For manual design, four primers were designed based on an alignment of different *cbsA* alleles. For primerBlast, unique Xtu genes were uploaded on primerBLAST for automatic primer design using a *Xanthomonas* database. **2**) Primer test and validation. To test the primers, three taxonomic groups of bacteria were used in colony multiplex PCR: non-*Xanthomonas* plant pathogens, *Xanthomonas* spp., and *X. translucens* pathovars. **3)** Field sample analysis. Barley field samples were used to test the primers. Pieces of plant material were incubated in sterile water to release bacteria. The liquid was plated on nutrient agar, and after two days growth, *Xanthomonas*-like colonies were selected for colony PCR.

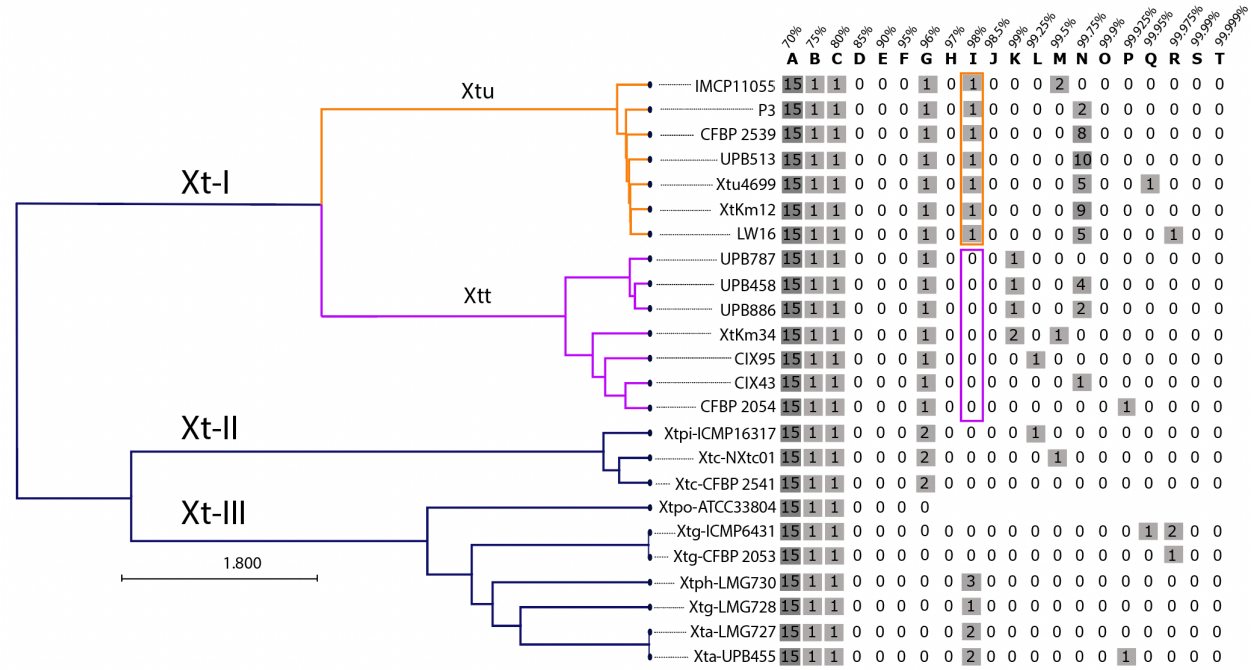

Figure 2. *Xanthomonas translucens* whole-genome average nucleotide identity (ANI) comparisons. The left panel is a phylogenomic tree generated with Ward as the clustering method based on Enveomics website results. The right panel is the Life Identification Number (LIN) classification. The percentages above letters A through T indicate the ANI values in each group. The genome of *X. translucens* pv. *poae* strain ATCC33804 has a short LIN because of large number of contigs present in the assembled genome, which does not allow a complete LIN classification analysis. In both panels, the orange and purple colors indicate the pathovar Xt-I-Xtu and Xt-I-Xtt, respectively. Xtc, *X. translucens* pv. *cerealis*; Xtpo, *X. translucens* pv. *poe*; Xtpi, *X. translucens* pv. *pistachiae*; Xtph, *X. translucens* pv. *phlei*; Xtg, *X. translucens* pv. *graminis; Xta, X. translucens* pv. *arrhenatheri.*

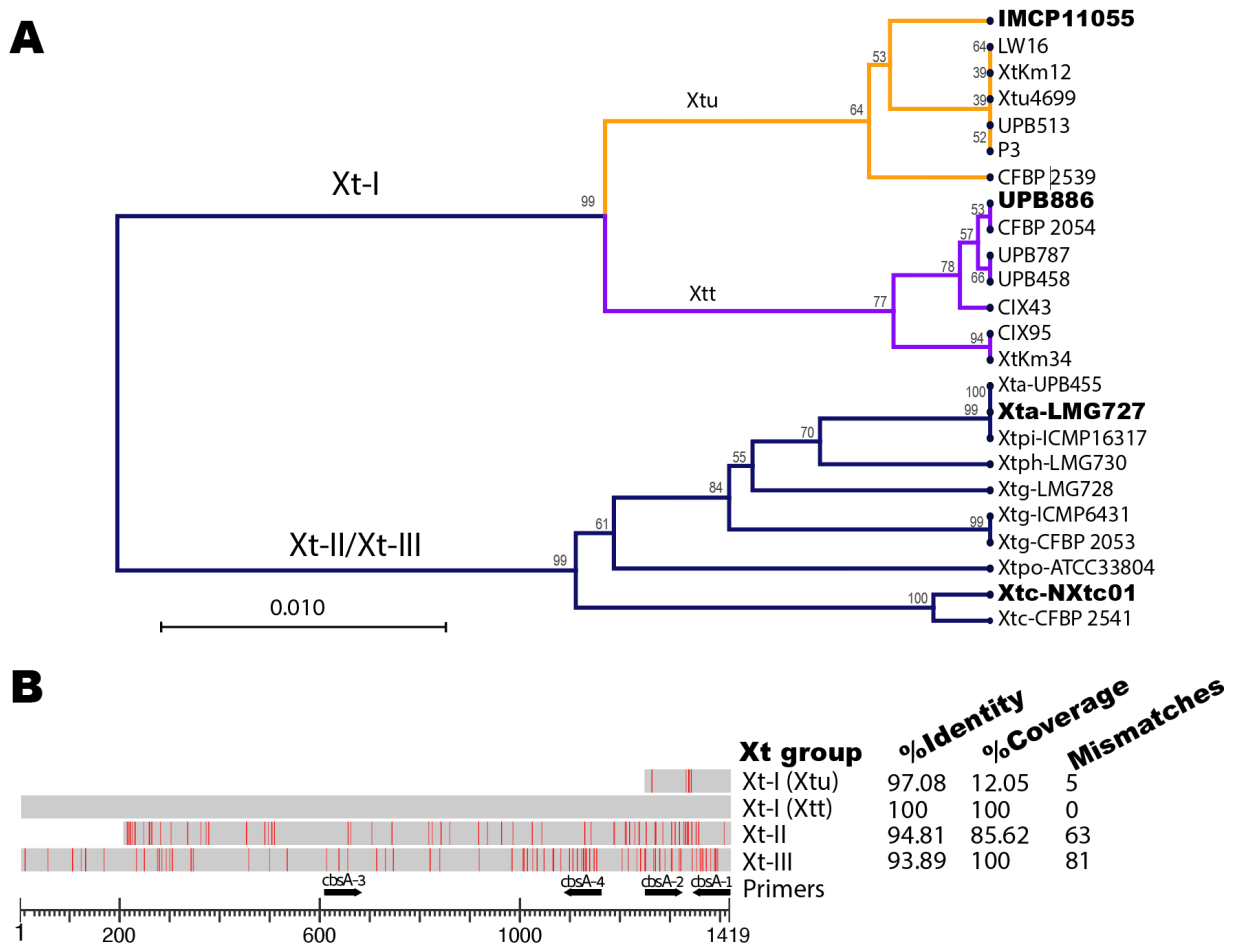

Figure 3. *cbsA* gene alignment using *X. translucens* genomes from NCBI. A) Simplified phylogram obtained from NCBI distance tree tool using default settings and edited using FigTree. Genomes in bold were used for the visual alignment. B) *cbsA* gene alignment from four representative alleles of *X. translucens* groups, two Xt-I strains ICMP11055 and UPB886, Xt-II strain NXtc01, and Xt-III strain LMG 727. The complete *cbsA* gene is 1429 bp while the truncated version is 165 bp long. The black arrows indicate the primer position in relation to the complete *cbsA*. Gray areas indicate conserved nucleotide regions while the red lines indicate nucleotide changes. The percent identity, percent coverage and mismatch values are based on a comparison to Xtt *cbsA* from strain UPB886. Alignment was made by NCBI Multiple Sequence Alignment Viewer 1.20.1 and edited in Adobe Illustrator. Xtc, *X. translucens* pv. *cerealis*; Xtpo, *X. translucens* pv. *poe*; Xtpi, *X. translucens* pv. *pistachiae*; Xtph, *X. translucens* pv. *phlei*; Xtg, *X. translucens* pv. *graminis; Xta, X. translucens* pv. *arrhenatheri.*

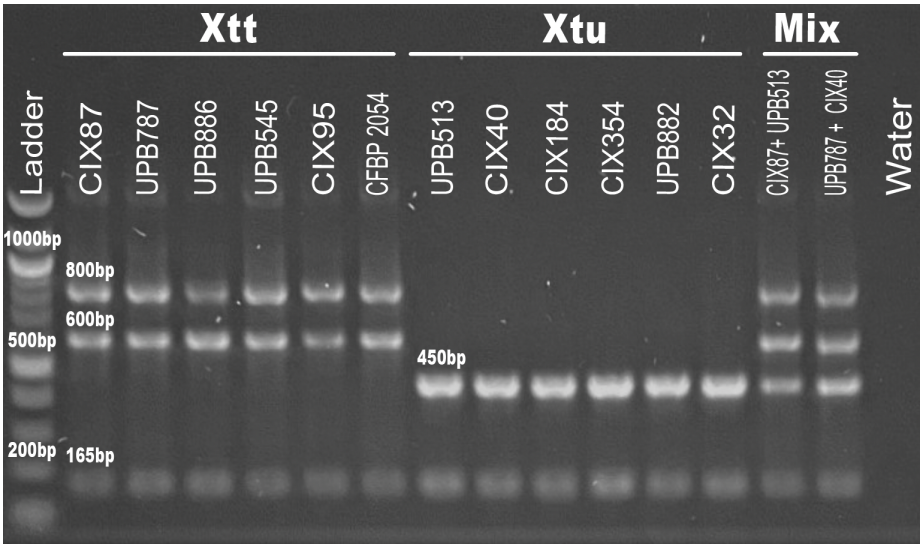

Figure 4. Multiplex PCR detection on single and mixed samples for *X. translucens* pv. *translucens* (Xtt) and *X. translucens* pv. *undulosa* (Xtu). Xtt strains are CIX87, UPB787, UPB886, UPB545, CIX95 and CFBP2054. Xtu strains are UPB513, CIX40, CIX184, CIX354, UPB882 and CIX32. The band profile for each group has a unique pattern, Xtt yields bands at 165 bp, 600 bp and 800 bp and Xtu yields bands at 165 bp and 800 bp. Mixed samples (Xtt CIX87+Xtu UPB513; and Xtt UPB787+Xtu CI40) have a combined profile presenting four bands. Amplicons shown on a 0.7% agarose gel with Quick-Load 100 bp DNA ladder (New England BioLabs Inc.)

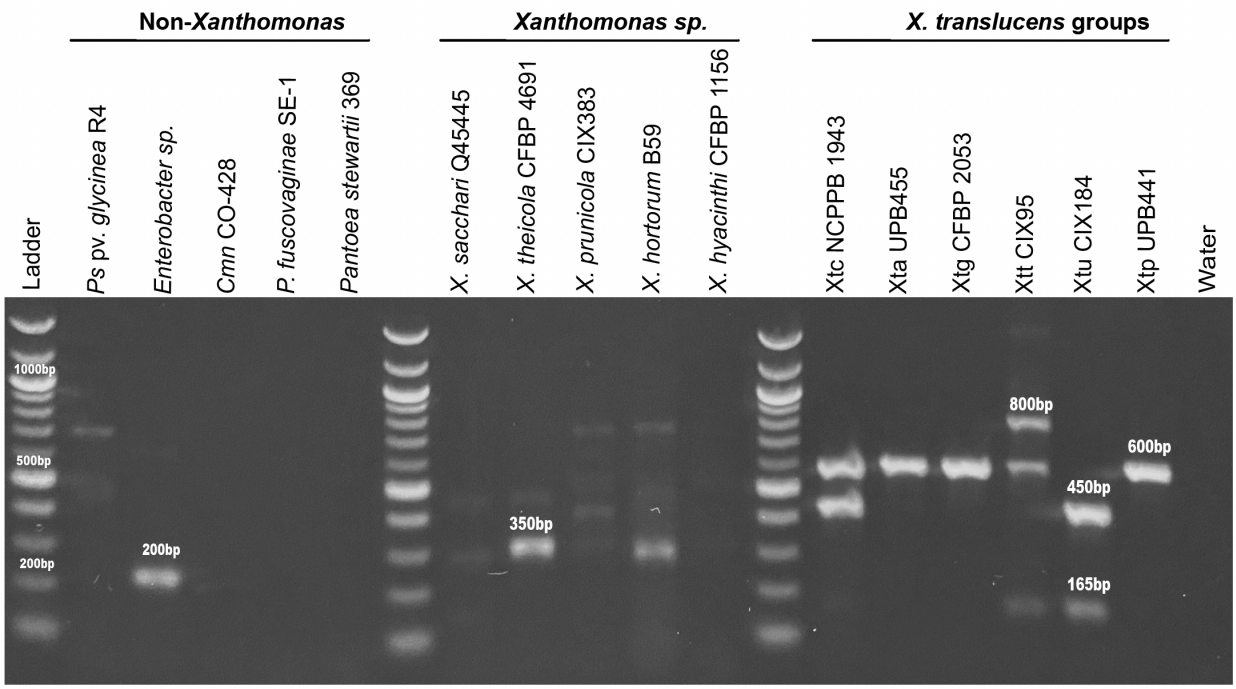

Figure 5. Multiplex PCR separates vascular and non-vascular pathogens in *Xanthomonas translucens* groups and can differentiate from non-*Xanthomonas* and other *Xanthomonas* species. Five non-*Xanthomonas* species were tested: *P. syringae* pv. *glycinea* (PsgR4); *Enterobacter spp.*; *C. nebraskensis* CO-428; *P. fuscovaginae* SE-1 and *Pantoea stewartii* 369. Five *Xanthomonas* species were tested: *X. sacchari* Q45445; *X. theicola* CFBP 4691; *X. prunicola* CIX383; *X. hortorum* pv. *vitians* B59 and *X. hyacinthi* CFBP 1156. Six *X. translucens* strains were tested: *X. translucens* pv. *cerealis* (Xtc) NCPPB 1943; *X. translucens* pv. *arrhenatheri* (Xta) UBP455; *X. translucens* pv. *graminis* (Xtg) CFBP 2053; *X. translucens* pv. *translucens* (Xtt) CIX95; *X. translucens* pv. *undulosa* (Xtu) CIX184; and *X. translucens* pv. *phleipratensis* (Xtp) UPB441**.** The gel is 0.7% agarose with Quick-Load 100 bp DNA ladder (New England BioLabs Inc.). The band size is indicated on the top of the distinguishing bands.

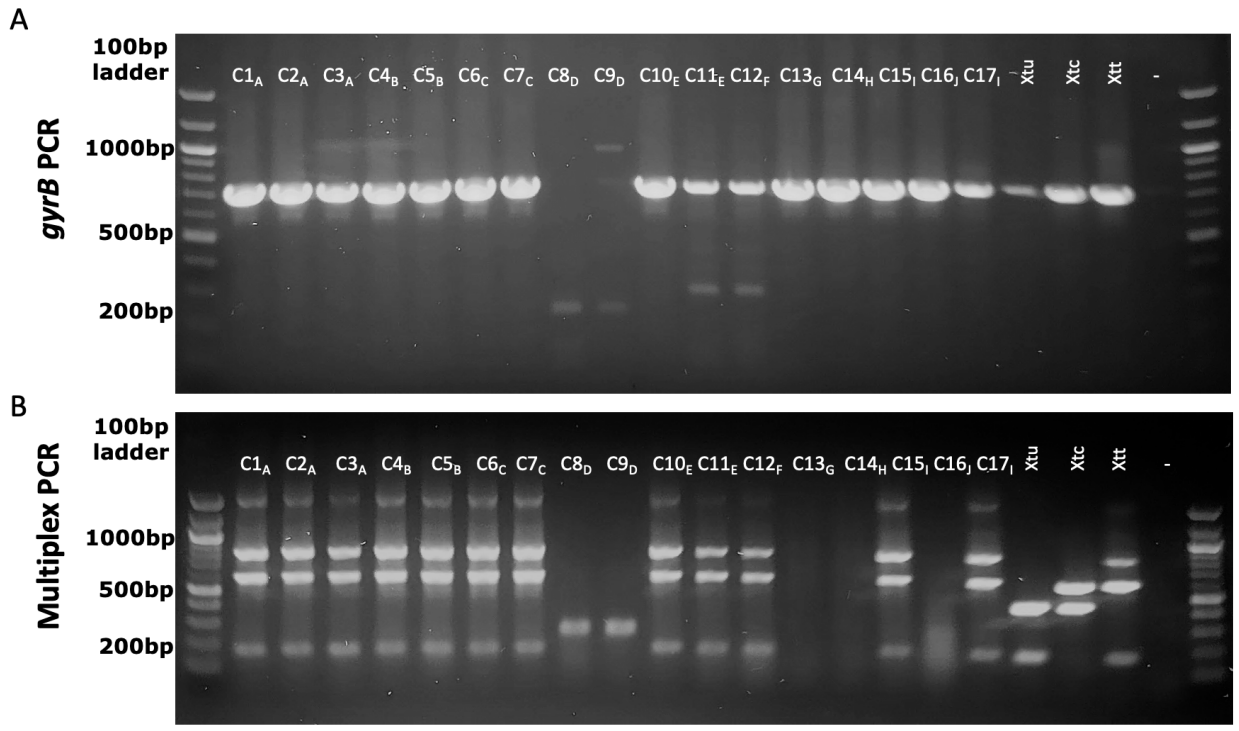

Figure 6. *Xanthomonas*-like *gyrB* and multiplex PCR. A) PCR reaction using *gyrB* primers (Parkinson et al. 2009). B) Multiplex PCR using *cbsA* 1-4 and S8.pep primers (Table 4). Seventeen colonies (C1-C17) isolated from ten different barley leaf samples (A-J) donated from growers. *X. translucens* pv. *undulosa* CIX184. *X. translucens* pv. *cerealis* NCPPB 1943. *X. translucens* pv. *translucens* CIX95 were used as control.

Table 1. List of non-*Xanthomonas* plant pathogens strains and *Xanthomonas* spp. strains tested with multiplex primers.

Species	Strain	Source
Pseudomonas syringae pv. *glycinea*	PsgR4 (PPY424)	Kobayashi et al. 1989
Enterobacter spp.	No strain recorded	Schwartz and Otto 2000
Clavibacter nebraskensis	CO-428	Lang et al. 2017
Pseudomonas fuscovaginae	SE-1	
Pantoea stewartii	369	Coplin et al. 2002
Xanthomonas sacchari	Q45445	Provided by APHIS
Xanthomonas hortorum pv. *vitians*	B59	Morinière et al. 2020
Xanthomonas hyacinthi	CFBP 1156	Cohen et al. 2020
Xanthomonas theicola	CFBP 4691	Koebnik et al. 2021
Xanthomonas prunicola	CIX383	Clavijo et al. 2021

Table 2. *Xanthomonas translucens* strains used to test multiplex primers.

Clade	Xt pathovar	Strain	Publication
Xt-I	*translucens*	CFBP 2054	Jones, Johnson et Reddy 1917
		UPB787 UPB886 UPB545	Bragard et al. 1997
		CIX87 CIX95	Curland et al. 2018; Ledman et al. 2021
Xt-I	*undulosa* *secalis*	CIX32 CIX40 CIX184 CIX354	Curland et al. 2018; Ledman et al. 2021
		UPB882 UPB513	Bragard et al. 1997
Xt-II	*cerealis*	NCPPB1943	Chaves and Mitkowski 2013
Xt-III	*phleipratensis* *graminis* *arrhenatheri*	UPB441 CFBP 2053 UPB455	Bragard et al. 1997 Pesce et al. 2015 Bragard et al. 1997

Table 3. Primer sequences for multiplex PCR.

Gene type *Gene name* RefSeq Sequence	Primer name: sequence (5'-3')	Annealing Temperature	Amplicon (bp)
Lifestyle-informed *cbsA* WP_003476231	*cbsA*-1: TCACTTTTCCGCTTGCTCGTTG *cbsA*-2: ATGTGCGACCCTACCTATAC *cbsA*-3: TGAGGAAGGCATCAAGTACGCA *cbsA*-4: CCTGGCTGTTTGACGAATGCAT	50°C	**Xtt**: 165 bp, 600 bp, 800 bp **Xtu**: 165 bp **Other Xt**: 600 bp
Xtu unique gene *S8 peptidase* WP_003469260	S8.pep-F: GACAGGGTCACGTTGGCATA S8-pep-R: CCTCCAATCAGTTCCCGGTC	50°C	**Xtu**: 450 bp

SUPPLEMENTARY MATERIAL

Table S1. Documented host range for *Xanthomonas translucens* pathovars.

Xt Clade	Pathovar	Host range	Reference
Xt-I	*translucens*	*Hordeum vulgare* *Hordeum maritimum* *Triticum* sp.	Jones et al. 1916; Bragard et al. 1997; Bradbury 1986) EPPO Code: XANTTR
	secalis/undulosa	*Triticum aestivum* *H. vulgare* *Secale cereale* *Bromus* sp. *Triticium durum* *Triticosecale* *Aegilops* sp. *Sesamum indicum*	Bragard et al. 1997; Rademaker et al. 2006; Reddy et al. 1924; Smith et al. 1919
		Asparagus virgatus	Rademaker et al. 2006
		Zizania palustris *Thinopyrum intermedium*	Curland et al. 2021, 2019
	pistachiae-A	*Pistacia vera*	Facelli et al. 2009
Xt-II	*cerealis*	*Bromus inermis* *H. vulgare* *T. aestivum* *S. cereale* *Avena* sp. *Elymus repens*	Rademaker et al. 2006; Wallin 1946; Bragard et al. 1997; Boosalis 1952; Hagborg 1974
	pistachiae-B	*Pistacia vera*	Giblot-Ducray et al. 2009
Xt-III	*graminis*	*Lolium* sp. *Festuca pratensis* *Phleum* sp. *Dactylis glomerata* *Trisetum* sp. *Lolium multiflorum*	Van den Mooter et al. 1987; Egli and Schmidt 1982
	poae	*Poaceae* (non-cereals) *Poa trivialis*	Bragard et al. 1997; Van den Mooter et al. 1987; Egli and Schmidt 1982
	phleipratensis /phlei	*Phleum pratense*	Wallin and Reddy 1945; fang et al. 1950

arrhenatheri	*Arrhenatherum elatius*	Egli and Schmidt 1982; Van den Mooter et al. 1987

Table S2. List of *Xanthomonas translucens* accession numbers for genome analysis.

***X. translucens* pathovar**	**Strain**	**NCBI accession**
undulosa	ICMP11055	SAMN03122553
undulosa	P3	SAMN10823695
secalis	CFBP 2539	SAMN18753387
undulosa	UPB513	SAMN05258786
undulosa	4699	SAMN02797519
undulosa	Km12	SAMN16598361
undulosa	LW16	SAMN10823694
translucens	UPB787	SAMN03168416
translucens	UPB458	SAMN18877146
translucens	UPB886	SAMN11831831
translucens	Km34	SAMN16598360
translucens	CIX95	CP072990
translucens	CIX43	CP072988-CP072989
translucens	CFBP 2054	SAMEA4358958
pistachiae	ICMP 16317	SAMN21172023
cerealis	NXtc01	SAMN11246188
cerealis	CFBP 2541	SAMN03246830
poe	ATCC 33804	SAMN05258173
graminis	ICMP 6431	SAMEA4071040
graminis	CFBP 2053	SAMN03892232
phlei	LMG730	SAMEA3486273
graminis	LMG728	SAMEA3486272
arrhenatheri	LMG727	SAMEA3486180
arrhenatheri	UPB455	SAMN04893617

Table S3. List of unique Xtu genes based on comparative genomes between Xtu and Xtt.

Gene name	GO annotation	Count
Avirulence protein AvrBs3	Plant-pathogen interaction	1
Azurin-1	Energy production and conversion	1
Cellulose synthase operon protein C	Cell wall/membrane/envelope biogenesis	1
Colicin V secretion/processing ATP-binding protein CvaB	Plant-pathogen interaction	1
Cytochrome c6	Amino acid transport and metabolism	1
Error-prone DNA polymerase	Replication, recombination, and repair	1
Hypothetical protein	Amino acid transport and metabolism	15
Hypothetical protein	Function unknown	33
IS4 family transposase ISLpn2	Replication, recombination, and repair	1
IS5 family transposase IS2000	Replication, recombination, and repair	1
LexA repressor	Transcription	1
Methyl-accepting chemotaxis aspartate transducer	Signal transduction mechanisms	1
Microbial serine proteinase	**Cell wall/membrane/envelope biogenesis**	1
Oxidoreductase UcpA	Function unknown	1
Probable transcriptional regulatory protein SgaR	Transcription	1
Protein ImuB	Replication, recombination, and repair	1
Protein oar	Inorganic ion transport and metabolism	1
Response regulator PleD	Signal transduction mechanisms	1
Transposase	Replication, recombination, and repair	1
Tyrocidine synthase 3	Secondary metabolites biosynthesis, transport, and catabolism	1
Uncharacterized transporter YxxF	Function unknown	1
Total number of unique Xtu genes		67

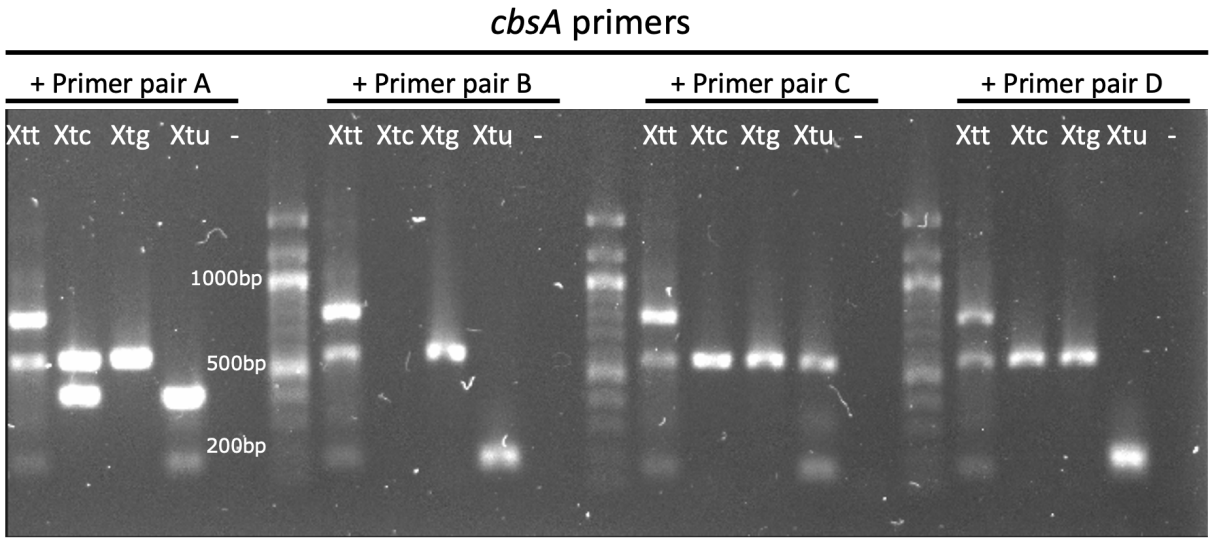

Figure S1. Multiplex PCR with *cbsA* primers and different Xtu primer pairs. Based on Table S2, primer pair A is microbial serine proteinase gene; primer pair B, is Azurin-1 gene; primer pair C, Tyrocidine synthase 3; primer pair D, Cellulose synthase operon protein C. Strains shown: *X. translucens* pv. *translucens* CIX95 (Xtt), *X. translucens* pv. *cerealis* NCPPB 1943 (Xtc), *X. translucens* pv. *graminis* (Xtg) CFBP 2053, *X. translucens* pv. *undulosa* CIX184 (Xtu).

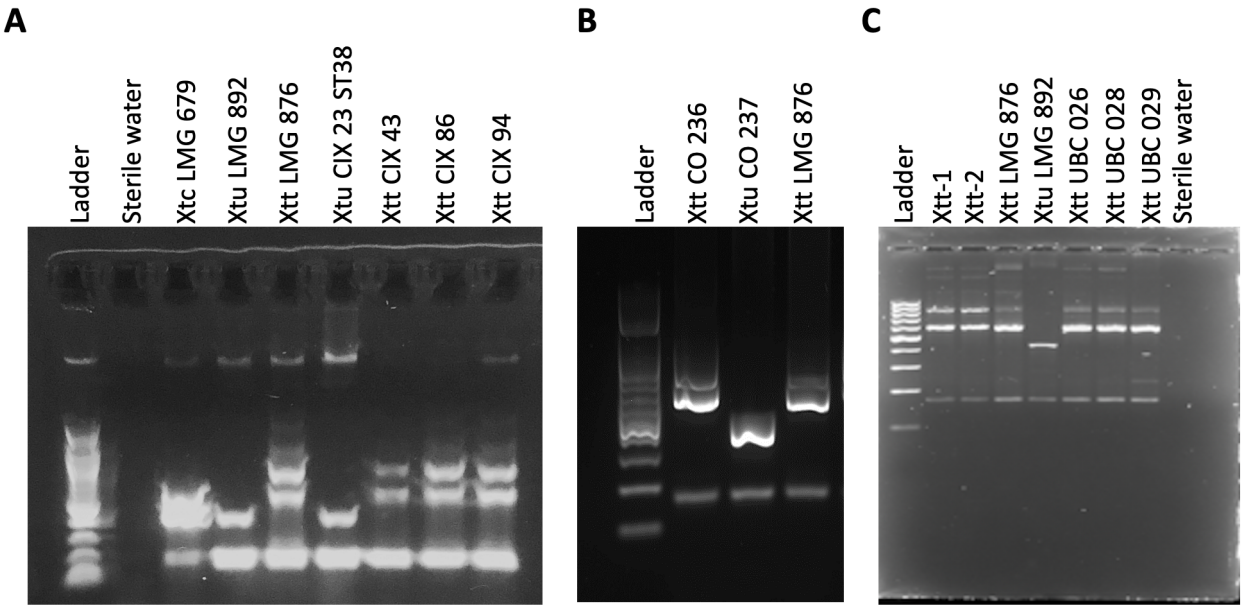

Figure S2. Gel electrophoresis showing results of *Xanthomonas translucens* multiplex PCR tested in three different laboratories. A) PCR results from University of Minnesota, B) PCR results from Colorado State University, C) PCR results from University of British Columbia.

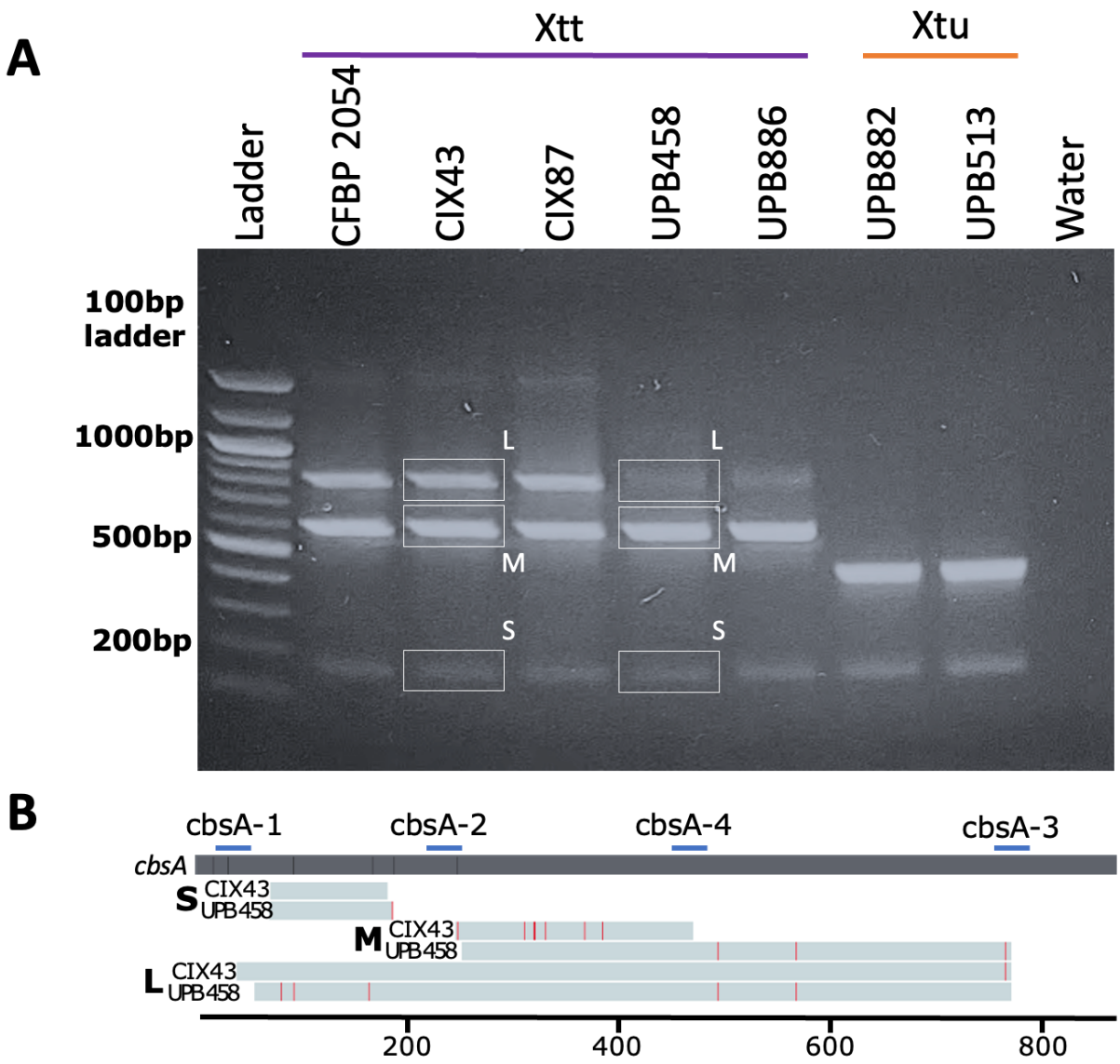

Figure S3. Sanger sequencing of the band profiles to confirm *cbsA* amplification. A) Band profile for several Xtu and Xtt strains. The bands were label based on the sizes, 165 bp (S), 600 bp (M), and 800 bp (L). The three bands for the Xtt strains CIX43 and UPB458 were Sanger sequenced. B) The sequenced fragments (light gray) were aligned to the *cbsA* gene (dark gray) from UPB 886 using the MAFFT algorithm. For reference, the four *cbsA* primers are indicated as blue lines. The red lines in the sequences indicate single nucleotide polymorphisms compared to the reference.