DEVELOPMENT OF QUANTIFICATION AND DIAGNOSIS METHODS FOR CEREAL BACTERIAL LEAF STREAK PATHOGENS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Bacterial leaf streak (BLS) has been a major disease on wheat and barley in the Northern Great Plains. It is caused by *Xanthomonas translucens* pv. *undulosa* (Xtu) on wheat and *X. translucens* pv. *translucens* (Xtt) on barley. Many questions remain unclear on pathogen biology and BLS epidemiology. Based on previous study, I identified an Xtu/Xtt specific region and established a qPCR quantification method for the bacterial pathogens. The method was shown to be effective to detect and quantify the bacterial pathogen in seeds and leaves. In addition, molecular markers were developed to differentiate Xtu and Xtt. Those markers were successfully used to characterize a collection of *X. translucens* strains into Xtu or Xtt. The results were also confirmed by pathogenicity tests on wheat and barley. The efficient Xtt/Xtu quantification and differentiation methods will be powerful tools to study disease epidemiology and host pathogen interaction for the two bacterial pathogens.

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GENERAL INTRODUCTION

Wheat and barley are the two most economically important cereal crops in the world. Wheat is a major source of calorie and other nutrients for the human being and barley is used as animal feed and as fermentable materials to make beer.

Bacterial leaf streak (BLS) is a common disease of wheat and barley worldwide. The disease is caused by *X. translucens* pv. *undulosa* (Xtu) on wheat and *X. translucens* pv. *translucens* (Xtt) on barley (Vautain et al., 1995). The disease can cause significant yield loss up to 40% as well as quality reduction in both wheat and barley (Waldron, 1929; Shane et al., 1987; Forster and Schaad, 1988). In the United States, the disease had been sporadic, mostly occurred on the warm and humid southern areas. In the last decades, the disease has become a major disease in the upper Midwest regions where the majority of US spring and durum wheat is grown (Duveiller et al., 1997; Adhikari et al., 2011; Kandel et al., 2012).

The control of BLS on wheat and barley is nearly impossible largely due to the very limited knowledge we have on the biology of the pathogen and disease epidemiology. Limited studies have been conducted in winter wheat regions and the results have shown that the bacterial pathogens can survive in seeds, crop residues, and nearby grass plants (Milus and Mirlohi, 1995; Duveiller et al., 1997; Stromberg et al., 2000). However, the major source of primary inoculum has not been determined. In addition, it remains unclear if the pathogens in spring wheat regions have same life cycles as those in winter wheat regions. To answer the unsolved questions on disease epidemiology and develop disease management methods, efficient bacterial pathogen detection and quantification tools are needed. Diagnostic molecular markers are available for *X. translucens* species and, some pathovars, but a quantification method for Xtt and Xtu pathovars has not been developed. Additionally, the two pathovars Xtt and Xtu have

different host range and tissue specificity, with Xtu able to cause the disease on wheat and barley on mesophyll tissue and Xtt able to cause the disease only on barley and vascular tissue (Jones et al., 1917; Smith et al., 1919; Hagborg, 1942; Vauterin et al., 1995; Duveiller et al., 1997; Adhikari et al., 2012; Gluck-Thaler, 2020). Little is known on how the two pathovars cause the disease in the two hosts in nature. For example, we do not know whether Xtu infects wheat and barley plants in field conditions. To answer this question, and others like it, we need to develop markers for detecting each pathovar.

Therefore, my thesis research had two objectives: 1) to develop a sensitive qPCR-based method for detection and quantification of Xtu/Xtt in different wheat and barley samples and, 2) to develop diagnostic DNA markers for Xtu and Xtt differentiations. The development of the molecular tools would help us to conduct further investigation into the biology of the pathogens, disease epidemiology, and host pathogen interactions.

LITERATURE REVIEW

Wheat production and classification

Wheat (*Triticum aestivum* L.) is the most extensively grown cereal crop all over the world and represents a main source of food (Oyewole, 2016). Wheat is one of the top three crops, along with corn and rice. The global population is 7.6 billion currently and is expected to reach 9.2 billion in 2050 (Food and Agriculture Organization and World Bank). The global wheat production needs to be increased by about 60-70% from the current level to meet the increased food demand in 2050. The global wheat production reached 775.9 million metric tons (MT) in 2020/2021 (Economic Research Service, USDA, updated on 10/12/2021). In the US, all wheat production totaled 1.65 billion bushels in 2021, down 10% from the 2020 total of 1.83 billion bushels (Small Grains 2021 Summary [September 2021] USDA, National Agricultural Statistics Service). Area harvested for grain totaled 37.2 million acres, up 1% from 2020 and the US yield was estimated at 44.3 bushels per acre, down 5.4 bushels from 2020 (Small Grains 2021 Summary [September 2021] USDA, National Agricultural Statistics Service). In fact, the wheat production in many major wheat-producing countries has been declining over the past two decades. The European Union and Russia have reduced wheat production by 10 million MT each year (Economic Research Service, USDA, updated on 9/14/2021). The US had exported around 25% of the global wheat between 2001 and 2005, but the number decreased to 13% for the 2020/21 marketing year (Economic Research Service, USDA, updated on 9/14/2021). Wheat ranks third, following corn and soybeans, among the U.S. field crops in planting acreage, production, and marketing. The states of North Dakota and Kansas are the two major producers of wheat in the U.S. In 2020/21, North Dakota had about 7.5 million acres of wheat planted with a total production of 320 million bushels.

Wheat grown in US is classified into five major groups including hard red winter wheat (HRWW), hard red spring wheat (HRSW), soft red winter wheat (SRWW), white wheat, and durum wheat. HRWW and HRSW constitute 60% of the wheat production in the US and are primarily used to produce bread flour. SRWW takes up 23% of wheat production and is used for the production of cakes, crackers, and cookies. White wheat has a wide range of characteristics and is favorited to make Asian-type noodles. Durum wheat is usually used for making pasta (USDA Economic Research Service. 6/24/2013).

Barley production and classification

Barley is also an important crop and ranks fourth globally in the total areas cultivated, following wheat, rice, and corn. The USDA estimated that the global barley production was 157.19 million MT in the 2020/2021 (Economic Research Service, USDA, updated on 01/09/2021). Barley is used for a variety of economic purposes. Over 50% of the barley produced in US is used for livestock feed. Barley is high in carbohydrates, with moderate amounts of protein, calcium, and phosphorus. Barley is also used for malting with about 80% in beer production, 14% in distilled alcohol production, 6% for malt syrup, malted milk, and breakfast foods. Barley is a cereal crop that belongs to the grass family Poaceae. There are three types of barley: *Hordeum vulgare* (the six-rowed type), *Hordeum distichum* (the two-rowed type), and *Hordeum irregulare* (the least cultivated).

Since the mid-1980s, barley production has decreased steeply throughout the US, from over 600 million bushels to 153 million bushels currently. North Dakota is a major barley producer with 90 % used for malting and brewing. The state experienced a sharp decrease in barley production between 2007 and 2017.

Bacterial leaf streak (BLS)

Bacterial leaf streak (BLS) is an important disease in wheat and barley worldwide. The disease was first reported in barley in 1917 (Jones et al., 1917) and in wheat in 1919 (Smith et al., 1919). BLS in wheat and barley are caused by *Xanthomonas translucens* pv. *undulosa* (Xtu) and *X. translucens* pv. *translucens* (Xtt), respectively (Adhikari et al., 2012; Curland et al., 2018). Similar diseases have been found on other cereal crops and forage grasses that are caused by different pathovars of this bacterium (see next section: "Symptoms and signs"). Historically, BLS epidemics have been reported to be sporadic and usually occur in warm and humid regions in the US. However, since 2008, BLS diseases of wheat and barley have become more important in the Midwest regions including North Dakota, South Dakota, and Minnesota (Adhikari et al., 2012; Kandel et al., 2012; Curland et al., 2018).

Symptoms and signs

The bacterium can infect both leaves and spikes, causing different BLS symptoms. During the early growth season, relatively short water-soaking streaks on the leaves are an important sign to identify BLS. The water-soaking streaks on the leaves can turn in irregular, elongated, and yellow to light brown-colored lesions, which can expand and coalesce to form large necrotic area in the leaves (Duveiller et al., 1997). Under warm and humid weather conditions, bacterial oozes can be observed within the lesions (Smith et al., 1919). The bacterial oozes contain bacterial cells that can splash to healthy leaves of the same plant or adjacent plants. The bacterium can also spread to the heads causing dark purple to black-colored streaks on the glumes, which is known as black chaff on wheat (Duveiller et al., 1997). Later in the growing season, BLS symptoms are difficult to be separated from those caused by fungal pathogens or environmental conditions, for examples, tan spot, Septoria nodorum blotch because

the lesions caused by those diseases become larger and coalesce to form large area of dead tissues. Detailed examination and molecule diagnosis tools would be required to distinguish between BLS and other fugal leaf spot diseases.

Geographical distribution

BLS disease occurs in many countries worldwide in wheat and barley-growing areas (Duveiller et al., 1997). Based on the European Plant Protection Organization (EPPO) Global Database, it has been reported in almost all continents, including North America (United States, Canada, and Mexico), South America (Brazil, Peru, and Argentina), Asia (China, Japan, India, and Iran), Africa (Ethiopia, Kenya, and Israel), Australia, and most parts of Europe (France, Russia, etc.). In most cases, the disease has been reported to be sporadic, but the epidemics of the BLS disease have occurred in several major wheat and barley-growing regions, including the Upper Midwest region of the United States where the majority of spring wheat and durum is produced (Adhikari et al., 2011; Kandel et al., 2012; Curland et al., 2020).

Disease cycle and epidemiology

Duveiller et al. (1997) proposed a disease cycle for BLS, but not much research has been done to confirm it, and many questions remain unanswered about some important steps. However, we know the bacteria can be detected in seeds harvested from an infected field and planting infected seeds can lead to disease epidemics in the field; thus, the infected seed is considered to be a source of primary inoculum (Foster and Schaad, 1988; Milus and Mirlohi, 1995). It has been suggested that seed lots with less than 1,000 CFU/g do not result in disease epidemics in field (Duveiller et al., 1997). However, the exact rate of bacterial transmission from infected seeds to the next crop has not been clearly defined. Furthermore, treating seeds with

bactericides seems to not eliminate disease in the field (Braun, 1920; Duveiller et al., 1997). It also remains unclear how the bacterium travels from the seeds to the above ground plants.

More specifically, Xtu has a wide host range, including smooth brome and quack grasses, and has demonstrated that the bacterial pathogen can grow epiphytically on other nonhost grass species (Wallin, 1946; Fang et al., 1950; Boosalis, 1952; Thompson et al., 1989). For example, recently Xtu has been identified in wild rice and common weedy grasses in Minnesota (Ledman et al., 2019; Curland et al., 2021). Thus, weedy grasses around wheat or barley fields could serve as a source of primary inoculum. However, the role that weeds play in disease epidemiology needs more study.

It has been also reported that the bacterial pathogens cannot survive very well in soil and/or crop annual crops debris, suggesting that crop residue is not a significant source of primary inoculum (Boosalis, 1952; Milus and Mirlohi, 1995; Duveiller et al., 1997). In addition, BLS is observed more often in areas with warm and humid conditions, suggesting that these conditions are favorable for BLS development. Since BLS tends to be sporadic and varied from year to year, we need more research on the weather factors that promote BLS epidemics.

Economic importance

Although BLS epidemics affect wheat and barley production areas worldwide, no recent reports are available on yield loss due to BLS. In a very early study, Waldron (1929) reported a reduction of 40% in thousand kernel weights in North Dakota spring wheat varieties. Sixty years later, studies indicated that BLS is an important limiting factor for grain yield in wheat and barley (Forster and Schaad, 1988; Duveiller et al., 1997). Currently, we know that yield losses have been reported to be 10% or less, but severe infections cause as high as a 40% yield loss from severely diseased fields in Idaho, in the western US (Forster et al., 1986; Duveiller et al.,

1997). Based on one field trial conducted by the NDSU extension service, BLS caused up to a 60% yield loss in highly susceptible varieties in North Dakota (Friskop et al., unpublished data). Sterile spikes due to BLS lead to extensive leaf necrosis for the whole plant (Duveiller et al., 1997; Tillman et al., 1999). In addition, BLS also affects the level of protein in grains, resulting in quality reduction (Shane et al., 1987). Therefore, the disease can be devastating to the entire nursery severely and, as a result, the harvest may become impossible, which can cause enormous economic damage (Burton, 1931).

Disease management

Currently, managing BLS is very difficult because not much research has been done and no effective methods are available. There are a few strategies to reduce BLS incidence available, such as using clean or pathogen-free seeds, bactericide application, and deployment of resistant varieties (Forster and Schaad, 1988; Duveiller et al., 1997). Because infected seeds serve as a source of primary inoculum, it is a logical idea to use pathogen-free seeds for planting. Some physical (heat) or chemical methods have been shown to reduce the bacterial concentrations in the infected seeds (Atanasoff and Johnson, 1920; Forster et al., 1990). However, these methods cannot completely eliminate bacteria in the seeds. Furthermore, because the bacteria cannot survive well in plant debris, crop rotation may not have much impact on BLS management (Duveiller et al., 1997). Copper-based bactericides have been successfully used for managing bacterial diseases on vegetables or tree crops (Schüder et al., 2004; Capinera and Dickens, 2016). In wheat, some copper-based bactericides have also been tested in field for plant protection from BLS, and preliminary data has shown inconsistent results for their efficiency (Friskop et al., unpublished data). Because of their simplicity and environmental benefits, resistant cultivars are the preferred way for controlling plant diseases. However, the majority of wheat cultivars

cultivated in this region are moderately to highly susceptible to BLS (McMullen and Adhikari, 2011) and only a few had partial resistance (see next section: "Identification and mapping of genetic resistance"). Planting cultivars with partial resistance could reduce the disease incidence and epidemics.

Identification and mapping of genetic resistance

Disease screenings have been conducted on a diversity of wheat germplasm from different sources to identify source of resistance for BLS under greenhouse conditions or field conditions (Hagborg, 1974; Akhtar and Aslam, 1986; Duveiller et al., 1993; Alizadeh et al., 1994; Milus and Mirlohi, 1994; El Attari et al., 1996; Milus et al., 1996; Tillman et al., 1996; Adhikari et al., 2011; Kandel et al., 2012; Falahi Charkhabi et al., 2017; Sapkota et al., 2018). The germplasm included wheat/barley cultivars, breeding lines, landraces, and related species. The results from many screening efforts showed that low percentage resistant accessions or lines are presented in wheat and barley germplasms, the majority of which have only partial resistance (Tillman et al., 1996; Adhikari et al., 2011; Kandel et al., 2012). The wheat lines identified with partial resistance included 'Pavon 76', 'Mochis 88', 'Thornbird' (Duveiller et al., 1993), 'Terral 101' (Milus and Mirlohi, 1994), 'Daqingshan No.3', 'Daqingshan No.4', 'MN 81319', 'GP 5012' (Tillman et al., 1996), and 'SD4205' (Kandel et al., 2012). Another problem with BLS resistance was likely environmentally dependent. Tillman et al. (1996) found inconsistent reactions for some of resistant lines among different conditions and different years, for example 'Pavon 76'. However, several triticale lines were identified to have high levels of resistance to BLS in greenhouse and/or field by several studies (Cunfer and Scolari, 1982; Johnson et al., 1987; Sapkota et al., 2018). These high levels of resistance would be very useful to improve BLS resistance in wheat.

The genetics and mapping of BLS resistance have been investigated in several studies by using classic genetic analysis and modern QTL analysis on the wheat lines with partial resistance (Duveiller et al., 1993; El Attari et al., 1996; Tillman and Harrison, 1996; Adhikari et al., 2012b; Kandel et al., 2015). The results mainly suggested a multigenic control and quantitative inheritance of BLS resistance. Duveiller et al. (1993) used classic genetic analysis to characterize resistance in three partially resistant varieties and identified a total of five genes (Bls1, Bls2, Bls3, Bls4, and Bls5 with Bls1) present in all three partially resistant wheat cultivars and having the largest effect. Tillman and Harrison (1996) studied resistance in 'Terral 101' and found that its inheritability was relatively low (average 0.31) and detected high levels of G × E interactions. Adhikari et al., 2012 used a GWAS approach and identified five genomic regions on chromosomes 1A, 4A, 4B, 6B, and 7D associated with BLS resistance. Kandel et al., 2015 mapped two QTLs on chromosomes 2A and 6B, respectively, conferring partial resistance in spring wheat breeding line 'SD4025' using identity by descent (IBD)-based QTL mapping methods. In barley, El Attari et al., 1998 identified three genomic regions associated with BLS resistance, two on chromosome 3H and one on 7H in the partially resistant barley line 'Morex'. Using classic genetic analysis, Johnson et al., 1987 studied the high levels of resistance in triticale line 'Syskiyou', which revealed a single gene, designed as Xct1, controlling resistance. Recently, Wen et al., 2018 successfully mapped Xct1 to the rye chromosome 5R using recombinant bred line triticale population. This provided a useful tool and information for transferring resistance gene Xct1 to wheat.

Xanthomonas translucens

X. translucens is a group of bacteria that cause diseases on cereals and grasses. The pathogen was first isolated from barley and named as *B. translucens* (Jones et al., 1917). Two

years later, a similar bacterial pathogen was isolated from wheat, which was named as *B. translucens* var. *undulosum* (Smith et al., 1919). After that, closely related bacterial pathogens usually found in wheat and barley were identified from other cereal crops, such as rye, triticale, oat, and many Poaceae grass species (Hagborg, 1942). Those phenotypically and genetically similar bacteria are collectively known as *X. translucens*. *X. translucens* is a gram-negative, motile and rod-shaped bacterium with a single polar flagellum and produces yellow and mucous colonies on nutrient agar media (e.x. Peptone sucrose agar, PSA) (Ou, 1985; Adhikari et al., 2011). Many nutrient agars and Wilbrink's agar media are not semi-selective and can be used for a wide range of bacteria (Sands et al., 1986). Several selective media including KM-1, XTS, and WBC have been developed for isolating *X. translucens* from seeds and leaf tissues (Duveiller et al., 1997).

Classification and nomenclature

The early taxonomy and nomenclature for *X. translucens* was mainly based on the host they were isolated from and the hosts they can infect by artificial inoculation. Due to high levels of morphological similarities and overlapping host ranges, the early taxonomy system for *X. translucens* has been very confusing. Dowson (1939) first established the *Xanthomonas* genus and the species of *X. translucen* that cause cereal BLS disease. Later, Hoagborg, 1942 accepted the genus *Xanthomonas*, but re-categorized *X. translucens* into five formae speciales (f. spp.): f. sp. *hordei* (barley), f. sp. *undulosa* (wheat, barley, and rye), f. sp. *secalis* (rye), f. sp. *hordei*-avenae (barley and oat), and f. sp. *cerealis* (wheat, barley, rye, and oat). Based on pathogenicity tests using inoculation, the five formae speciales (from Hoagborg, 1942) were distinguished as f. sp. *hordei* (barley), *undulosa* (wheat), *secalis* (rye), *cerealis* (B. inermis), and *phleipratensis* (timothy). F. sp. *cerealis* was given its name as a strain that naturally occur on smooth

bromegrass and quack grass but can infect wheat, barley, rye, and oat (Fang et al., 1950). Dye and Lelliott (1974) classified all *X. translucens* on cereals into the species of *X. campestris* as different pathovars, which included *translucens, undulosa*, *cerealis, hordei,* and *secalis*. Accordingly, the bacterial pathogens causing bacterial wilt in forage grasses (which is closely related to cereal *X. translucens*) were also placed in to *X. campestris* as pathovars, including *X. campestris* pvs. *graminis, phlei, poae,* and *arrhenatheri* (Egli and Schmidt, 1982; Van den Mooter et al., 1987).

Since the late 1980s, studies using biochemical and molecular analyses, such as protein electrophoresis, fatty acid gas chromatography, and DNA-DNA hybridization, have revealed a large amount of heterogeneity within the *X. campestris* group (van den Mooter, 1987; Azad and Schaad, 1988; Kersters et al., 1989; Vauterin et al., 1992). These studies led to a reclassification of the genus *Xanthomonas* (Vauterin et al. 1995). Vauterin et al. (1995) reclassified the species *X. translucens* to include all the *Xanthomonades* in cereals and forage grasses and separated the *X. translucens* into two main groups with the "translucens group" including pathovars causing BLS on cereals (pvs. *undulosa*, *translucens*, *cerealis*, *hordei*, and *secalis*) and the "graminis group" including pathovars causing disease on forage grasses (pvs. *graminis*, *poae*, *phlei*, and *arrhenatheri*) (Vauterin et al., 1992). At the time, the pathovars causing BLS in cereal crops were considered to be closely related, as revealed by using DNA-DNA hybridization, protein electrophoresis, fatty acid profiling, and 16S rDNA sequencing (Stead, 1989; Vauterin et al., 1992; Hauben et al., 1997). However, more sensitive genetic and biochemical methods such as gas chromatography of fatty acid methyl esters (FAME), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins, and amplified fragment length polymorphism (AFLP) have revealed three biological entities, which were classified into three pathovars: pvs.

undulosa, *translucens*, and *cerealis* (Vauterin et al., 1992; Yang et al., 1993; Bragard et al., 1995).

This classification has also been supported by the evidence from genome sequence data. Langlois et al. (2017) sequenced 15 *X. translucens* strains and developed a phylogenetic tree containing two main clades of non-cereal and cereal *X. translucens*. Within the cereal group, pv. *cerealis* strains were separated into a subclade that was distinct from the cereal group comprised of the two subclades Xtu and Xtt (Langlois, 2017). Peng et al. (2016) also revealed three different pathovars: pvs. *undulosa*, *translucens* and *cerealis* of *X. translucens* on cereal crops.

Genome sequences

The first genome sequence for *X. translucens* was conducted from *X. translucens* pv. *graminis* strain Xtg29 (Wichmann et al., 2013). Among 51 *X. translucens* genome assemblies in the NCBI genome GenBank, three strains were completely sequenced with long-reads sequencing techniques, including two Xtu strains Xt4699 from the US and ICMP11055 from Iran, and one Xtt strain DSM 18974 (Jaenicke et al., 2016; Peng et al., 2016; Charkhabi et al., 2017). The rest of the strains in the *X. translucens* group were sequenced with short-reads platforms. The annotated protein-encoding genes in *X. translucens* genomes ranged from 3,160 to 4,413. Currently, the complete genome sequences of available Xtu strains and Xtt strains were generated by Oxford Nanopore or Pacific Biosciences (PacBio) or Illumina (Peng et al., 2016; Falahi et al., 2017; Roman-Reyna et al., 2020). The Xt4699 was the first completely sequenced Xtu strain; it was collected in Kansas, US and consists of 4,561,137 bp (Peng et al., 2016).

Pathogenicity and virulence

Plant pathogenic bacteria employ diverse pathogenicity and virulence factors for successful infections and subsequent multiplications in the host plants (Wilson, 2002; Wu et al.,

2008; Buttner and Bonas, 2010). The biological functions of these pathogenicity or virulence factors can disrupt host cell components or tissues directly, protect the bacteria from recognition, or suppress host immune systems (Wilson, 2002; Wu et al., 2008). In fact, the Type III secretion system (T3SS) and the effector proteins secreted through T3SS, known as T3Es, are essential for pathogenicity and virulence of most gram-negative plant pathogenic bacteria (Ghosh, 2004; Peng et al., 2016; Shah et al., 2019). T3SS is encoded by the chromosomal hrp (hypersensitive response [HR] and pathogenicity) gene cluster, which contains more than 20 genes organized in several transcriptional units (Buttner and Bonas, 2002; White et al., 2009). T3SS is a syringe-like structure spanning bacterial cell membranes and plant cell membrane and used by the bacteria to inject effector proteins into plant cell cytoplasm.

Each *Xanthomonas* strain produces ~30 effector proteins, known as Xanthomonas outer proteins (Xops), to manipulate diverse plant cell biological processes for bacterial benefits (Roden et al., 2004). *Xanthomonas* has a unique group of T3Es; these are called transcription activator-like effectors (TALEs) because after secretion into plant cytoplasm they enter further into the nucleus and function like a transcription activator. TALEs recognize specific DNA sequences, called effector-binding elements (EBEs) in the promoter region of a host gene. The transcription of the particular gene or genes leads to either susceptible or resistant reaction (Atef, 2020). TALEs usually have an N-terminal domain, including the type III secretion signal, a central repeat region (CRR) interacting with host EBEs, a C-terminal domain containing nuclear localization signals, and an acidic activation domain (Boch and Bonas, 2010; Mak et al., 2013). The number of tal genes is highly variable in *Xanthomonas* spp. For example, there are eight tal genes in strains of *X. translucens* pv. *translucens*, seven in pv. *undulosa*, and two in pv. *cerealis* (Pesce et al., 2015; Jaenicke et al., 2016; Peng et al., 2016; Charkhabi et al., 2017). Several

TALEs have been shown to play a significant role in virulence in Xtu and Xtc strains (Charkhabi et al., 2017; Peng et al., 2019; Shah et al., 2019). Very interestingly, Peng et al. (2016) showed that the bacterial pathogen used Tal6 in Xt4699 to activate ABA synthesis genes in wheat plants, resulting in bacterial growth enhancement.

Bacterial detection and quantifications

Detection and identification of bacterial pathogens in crops are very important to decrease disease-induced crop damage during growth, harvest, and postharvest processing, as well as to increase crop productivity (Fang and Ramasamy, 2006). The traditional detection and identification methods were mainly based on bacterial culturing on selective media and a series of biochemical testing (Franco-Duarte, 2019). Later, molecular detection methods were developed that are faster, more accurate, and more high-throughput. Furthermore, there are a variety of molecular detection methods available, such as conventional Polymerase Chain Reaction (PCR), Real-time Quantitative PCR (qPCR), Enzyme-Linked Immunosorbent Assay (ELISA), Loop-Mediated Isothermal Amplification (LAMP) assay, and Recombinase Polymerase Amplification (RPA). In recent years, genome and 16s rDNA amplicon sequencing have been used for a wide range of microbial profiling in microbiome studies (Regalado, 2020).

Conventional PCR

Conventional PCR is the most common method for bacterial pathogen detection (Cai et al., 2014). PCR assays require a set of primers specific to the targeted pathogen and have a series of processes such as DNA denaturation, annealing, and extension for DNA amplification. The amplified PCR products are either present or absent, revealed by electrophoresis in an agarose gel. The primer sets are usually designed based on the specific sequence of 16s rDNA or inter-transcriptional spacer (ITS) region or other genomic regions (Maes et al., 1996; Liguori et al.,

2011). This method is particularly useful for difficult-to-culture or unculturable pathogens, or for those that are difficult to characterize using other traditional methods (Cai et al., 2014). In addition to the basic PCR technology, multiplex PCR have also been developed for plant pathogen identification due to their high sensitivity (Yi and Ramaraja, 2015).

Quantitative PCR (qPCR)

Quantitative PCR (qPCR) is derived from conventional PCR with a specific primer set to amplify a shorter fragment. qPCR can be used for both detection and quantification purposes with a great level of sensitivity. During the reactions, the PCR products are monitored in real-time in each cyclic repetition of amplification by the use of a fluorescent dye or a fluorescein-labeled probe. After several PCR cycles, the level of fluorescence emitted rises to a baseline called the cycle threshold (Ch) value (Grosdidier, 2017). In data analysis, software (Bio-Rad Laboratories, Inc.) allows the baseline threshold to be set for each target to calculate Cq value. If a standard curve is present, the CFX software will automatically calculate reaction efficiency and show amplification traces, melt peak data, a full table of quantification results, and standard curve information. The Cq value is highly correlated with the initial DNA template concentration, with low Cq indicating high concentration and high Cq indicating low concentration. Thus, qPCR can be used to quantify the targeted organism. However, qPCR can be affected by several factors, such as the brand of qPCR reagents, the quality of extracted DNA, qPCR equipment, and analytic software (Freeman et al., 1999). In addition, as the number of qPCR cycle increases, the possibility to obtain late Cq values generated by non-specific amplification also increases (Pfaffl, 2004). The qPCR detection methods are applicable to other plant diseases, for example Stewart's wilt of corn, caused by the seed-born bacterium *Pantoea stewartia* subsp. *stewartia* (Pal et al., 2019).

ELISA assay

ELISA is a molecular method for pathogen detection and identification based on antibodies and color change in the assay (Clark and Adams, 1977). In this method, the target epitopes (antigens) from the viruses, bacteria, and fungi specifically bind with antibodies conjugated to an enzyme. In the final step, as a substance containing the enzyme's substrate is added, if there is binding, the subsequent reaction produces a detectable signal, most commonly a color change. Thus, the detection can be visualized based on color changes. However, the sensitivity for bacteria is relatively low (10^5-10^6 CFU/mL), making it useful only for the confirmation of plant bacterial pathogenic bacteria after visual symptoms appear.

LAMP assay

LAMP assay was first described by Notomi et al. (2000) and it is a sensitive and fast detection method for a diverse range of plant pathogens. The LAMP assay amplifies nucleic acids with a set of four specific primers: two inner primers (FIP and BIP) and two outer primers (F3 and B3), in combination with the standard displacement activity of DNA polymerase (Duan et al., 2014; Kaczmarek et al., 2019). If additional loop primers are included, the LAMP assay can make detection faster and more sensitive (Nagamine et al., 2002). LAMP products can be visualized with the naked eyes by adding DNA-intercalating dyes such as ethidium bromide, SYBR Green I, propidium iodide, or Quant-iT PicoGreen (Duan et al. 2014). LAMP products can also be detected by real-time detection method (Bekele, 2011; Langlois et al., 2017).

RPA

RPA is an isothermal DNA amplification technology developed by Piepenburg et al., 2006. RPA is designed to be performed in field conditions where no special equipment is available. It is a versatile alternative to polymerase chain reaction (PCR) for the development of

simple, fast, portable, nucleic acid detection assays working at a low temperature with lyophilized reagents without the process of DNA denaturation.

RPA does not need the extra step for template melting, which allows the primers to bind to their complementary target sequences. Instead, RPA uses recombinase-primer complexes to recognize double-stranded DNA, making strand exchange at cognate sites possible (Yonesaki, 1985). Single-stranded DNA binding (SSB) protein binds to the dislodged DNA strand, thus stabilizing the resulting D loop (Harris, 1988). Then recombinase disassembles, leaving the 39 bp end of the oligonucleotide accessible for a strand displacing DNA polymerase (e.g., the large fragment of *Bacillus subtilis* Pol I, Bsu) to extend the strand in the presence of dNTPs (Piepenburg, 2006; Lobato et al., 2018). Cyclic repetition of this process facilitates an exponential amplification (Piepenburg, 2006).

Detection methods for *X. translucens* pathogens

Characterization of *X. translucens* pathogens have been done using various methods, such as DNA-DNA hybridization, membrane protein assay, biochemical and serological tests, culturing on semi-selective media, and pathogenicity tests (Azad and Schaad, 1988; Bragard et al., 1995; Elrod and Braun, 1947; Vauterin et al., 1995; Rademaker et al., 2006). The first PCR-based detection method was developed by Maes et al., 1996 using a ribosomal DNA spacer region (Maes et al., 1996). The primers T1/T2 were designed based on the sequence the 16S and 23S rRNA genes, which produced a 139 bp DNA fragment from all *X. translucens* pathogens, but not in other *xanthomonads* (Maes et al., 1996). The method was successfully applied in the detection of bacterial pathogens in infected field seeds. However, the primer set cannot distinguish different *X. translucens* pathovars from each other (Maes et al., 1996). Langlois et al. (2017) reported several genome sequences in *X. translucens* and the use of comparative

genomics to identify genomic regions that are specific to individual subgroups within *X. translucens*. Based on these specific genome regions, the authors developed different sets of LAMP primer that can be used to detect specific groups. The primers included Xt-CLS specific to cereal leaf streak pathogens, a forward inner primer (FIP), a backward inner primer (BIP), a forward outer primer (F3), and a backward outer primer (B3). The LAMP assay with Xt-CLS primer set was successfully established to detect the Xtu from the infected wheat seeds. Although diagnostic methods have been developed for Xtu and Xtt, the bacterial quantification method is not available for these two important cereal bacterial pathogens. In addition, the method to separate Xtu and Xtt is also absent.

DNA marker development

Genetic variations at a specific DNA sequence can be used to develop molecular markers to differentiate individuals. The major types of DNA-based markers include restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), and single-nucleotide polymorphism (SNP) (Powell et al., 1996; Jiang, 2013; Nadeem, 2018). RFLP was the first generation of DNA marker techniques (Tanksley, 1989). DNAs are first digested with a specific restriction enzyme and then blotted to a nylon membrane. A probe of interest is hybridized to the membrane to reveal the length polymorphisms for a specific cut fragment. RAPD was developed in 1990 (Williams et al., 1990; Welsh and McClelland, 1990). Genomic DNAs are amplified using PCR with different pairs of single, short oligo primers (about 10 bp). Because these short primers can bind to many loci in the genome, multiple bands can be amplified, some of which may be polymorphic between different individuals.

SSRs (or microsatellites) are pieces of DNA sequences containing simple motifs ranging from 1 to 6 nucleotides arranged as tandemly repeats; they have been widely distributed in eukaryotes genomes (Tautz and Renz, 1984; Tautz, 1989; Vogt, 1990). SSR markers have various benefits such as co-dominant inheritance, genome-wide coverage, high polymorphism with multiple alleles per locus, high reproducibility, and transferability between species. Therefore, SSR markers have been widely applied for fingerprinting, association mapping, and linkage mapping (Gyawali et al., 2016; Zhao et al., 2017).

SNP refers to variation at a single nucleotide position in the genome. SNPs are the most abundant source for DNA marker development. It is estimated that SNP frequency in plants can reach one SNP in every 100-300 bp (Xu, 2010). In addition, SNPs are widely distributed within the genome and can be found in coding or non-coding regions of genes or between two genes (intergenic region) with different frequencies (Xu, 2010). SNPs can be developed into DNA markers using various methods, such as cleaved amplified polymorphic sequence (CAPS), Kompetitive Allele-Specific PCR (KASP) (Majeed 2019), RNase H-dependent PCR (rhPCR, Dobosy et al., 2011) and semi-thermal asymmetric reverse PCR (STARP, Long et al., 2017). In the CAPS technique, target DNAs containing a specific restriction site at the SNP position are amplified using specific primers followed by digestion with the specific restriction enzyme and visualization of digested fragments (Jarvis et al., 1994; Michaels and Amasino, 1998). In my research, I developed CAPS markers to separate Xtu and Xtt.

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A QPCR-BASED METHOD TO QUANTIFY BACTERIAL LEAF STREAK PATHOGEN IN SEEDS AND LEAVES OF WHEAT AND BARLEY[1]

Abstract

Bacterial leaf streak (BLS) has become a major disease on wheat and barley in the Northern Great Plains. They are caused by *Xanthomonas translucens* pv. *undulosa* (Xtu) on wheat and *X. translucens* pv. *translucens* (Xtt) on barley, respectively (Vautain et al., 1995; Adhikari et al., 2012; Kandel et al., 2012; Curland et al., 2018). Seed is considered to be an important source of primary inoculum for these bacterial pathogens (Foster and Schaad, 1988; Milus and Mirlohi, 1995). In this study, a qPCR-based method was developed to quantify the pathogens in the seeds and leaves of wheat and barley. Using published information, a genomic region that is only present in the two pathovars was used. The two primers (F3/B3) reported in the previous study was confirmed to specifically amplify a band from all Xtu/Xtt strains tested, but not in other pathovars. A SYBR green based-qPCR method was developed by using F3/B3 to increase detection sensitivity and perform bacterial quantification. Standard curve assay with pure bacterial culture showed that the qPCR method can detect as low as $1.2x10^4$ colony-forming unit (CFU)/mL. To validate the qPCR method, the seeds of ten hard red spring wheat varieties harvested from field experiments were used for bacterial quantification tests. The results showed the bacterial concentration in these seeds ranged from $5.6x10^5$ to $5.1x10^7$ CFU/g. Statistical analysis revealed a weak correlation between bacterial concentration and BLS disease score. The method was also successfully applied to quantify the bacterial pathogens in wheat leaves and barley seeds.

[1] The material in this chapter was co-authored by Eunhye Hong, Dr. Zhaohui Liu, Dr. Andrew Friskop, and Dr. Rebecca Curland. Eunhye Hong conducted the experiments and drafted this chapter. Dr. Friskop provided us seed samples for the experiments. Dr. Curland Rebecca gave genomic DNA strains for the experiments. Dr. Liu served as proofreader.

This is the first report of a qPCR method for detecting and quantifying the important bacterial pathogens in wheat and barley and the method would be useful for seed certification and pathogen biology and disease epidemiology study.

Introduction

Bacterial leaf streak (BLS) is a common disease on a wide range of cereal crops and forage grasses worldwide. These diseases are caused by a group of related bacteria, collectively known as *Xanthomonas translucens*, which are further divided into different pathovars (Dye and Lelliott, 1974; Vauterin et al., 1995). Among them, *X. translucens* pv. *undulosa* (Xtu) and *X. translucens* pv. *translucens* (Xtt) are the two important pathovars causing disease on wheat and barley, respectively (Duveiller et al., 1997; Adhikari et al., 2012; Kandel et al., 2012; Curland et al., 2018). BLS has established to be a major disease in many wheat- and barley- growing regions including the Upper Midwest region of the United States (Adhikari et al., 2012; Kandel et al., 2012; Curland et al., 2018; Sapkota et al., 2020).

The early BLS symptoms on leaves are characterized by water-soaking streaks which then increase for length and width and further turn into irregular, elongated, and yellow to light brown-colored lesions. The lesions can continue to expand and coalesce to form large necrotic area on leaves (Duveiller et al., 1997). Under warm and humid conditions, yellow bacterial oozes can be observed along the water-soaking streaks on the leaves (Smith et al., 1919). Those bacterial oozes contain thousands of bacterial cells which can splash onto upper leaves, heads, and adjacent plants during the growing season. The infection on spikes by the bacterial pathogens can lead to the development of black or dark purple-colored lesions on the glumes, also called black chaff disease (Duveiller et al., 1997; Tillman et al., 1999). Since the BLS symptoms are similar to those of some fungal diseases, for example, Septoria nodorum glume

blotch, as well as stresses from environmental conditions, it is difficult to differentiate them in the field, particularly in the late growth season (Duveiller et al., 1997; Adhikari et al., 2011).

Although BLS has become a major problem in wheat and barley productions, limitedstudies have been done to investigate yield losses induced by BLS. Based on a few old studies, yield losses due to BLS could be 10% or less under low disease pressure, but severe infection has caused a 40% yield loss on highly susceptible varieties (Forster et al., 1986; Duveiller et al., 1997). Waldron (1929) reported a yield reduction of 40% in spring wheat varieties in North Dakota. BLS can also affect the protein level of grains, resulting in quality alterations (Shane et al., 1987).

Currently, managing BLS diseases is very difficult largely due to the limited knowledge on the biology of disease cycle and epidemiology. The bacterial pathogens are known to survive in the seeds which may serve as a source of primary inoculum (Foster and Schaad, 1988; Milus and Mirlohi, 1995), but it is unknown what levels of bacterial number in seeds are important for the disease initiation in the field (Duveiller et al., 1997). Boosalis (1952) reported that the bacterial pathogens cannot survive in crop residues and soil very well, suggesting crop residues is not a significant source of primary inoculum (Duveiller et al., 1997). Both Xtt and Xtu have been successfully isolated from grass species and other hosts, which suggested those hosts can be a repertoire of primary inoculum (Wallin, 1946; Fang et al., 1950; Boosalis, 1952; Thompson et al., 1989; Ledman et al., 2020; Curland et al., 2020). However, questions still remain to be answered on the exact role of those factors, such as crop residues, grass species in disease epidemics. To answer those questions, an effective detection and quantification method for those bacterial pathogens are needed.

Maes et al. (1996) was the first to develop a detection method for *X. translucens.* The method employed the conventional PCR with a primer pair (T1/T2) that was based on intergenic transcription spacer (ITS) region and can amplify a 139 bp fragment from all *X. translucens* pathovars (Maes et al., 1996). A recent study was conducted to develop a loop-mediated isothermal amplification (LAMP) assay for each group of *X. translucens* from the whole genome sequencing and comparison among 15 *X. translucens* strains (Langlois et al., 2017). These methods were capable of detecting Xtu in the wheat seeds (Maes et al., 1996; Langlois et al., 2017). However, a sensitive method to quantify Xtu and Xtt in plant tissues is still not available. Therefore, the objectives of my study were 1) to develop a SYBR green-based qPCR method for Xtt and Xtu quantification 2) to determine bacterial concentrations in various wheat and barley tissues by using the developed qPCR method.

Materials and methods

Bacterial strains and culturing

A total of 77 bacterial strains was used in the testing of the qPCR primer specificity, including 44 Xtu and 23 Xtt, six strains from *X. translucens* 'graminis group', and three unrelated bacterial species. The Xtu and Xtt strains were mainly collected from North Dakota (four different locations: Langdon, Lisbon, Casselton, Carrington) and Minnesota. ND strains were maintained in our lab and MN strains were kindly provided by Dr. Rebecca Curland at the University of Minnesota (UMN). The 'graminis' group strains included *X. tranclucens* pvs. *graminis, poae, phlei, phleipratensis, cerealis, and arrhenatheri,* which were kindly provided by Dr. Rebecca Curland at UMN. The unrelated bacterial strains included *E. coli* K-12, *X. axonopodis* pv. *sojense*, and *Agrobacteria tumerfaciens,* which were kindly provided by Dr. Julie Pasche's group in our department. The Xt4699 strain was kindly provided by Dr. Frank White at

Kansas State University. All the bacterial strains were cultured on Wilbrink's Agar (WBA) medium (Bacto Peptone 0.5%, sucrose 1%, K_2HPO_4 0.05%, $MgSO_4.7H_2O$ 0.025%, Na_2SO_3 (anhydrous) 0.005%, and agar 1.5%). The culture was usually incubated at 28 ºC for 2 days for colonies to fully form and for DNA extractions.

Bacterial genomic DNA extraction

Genomic DNA was isolated from the ND bacterial strains using the extraction method described in Richards et al. (2018). The DNA concentrations were measured using Nanodrop one UV-Vis spectrophotometers (Thermo Fisher Scientific) and was adjusted to 5-6 ng/µl for PCR. Genomic DNAs of other bacterial strains such as Xtt strains from MN, 'graminis group', and unrelated bacterial species were directly supplied by the providers.

Confirmation of a genomic region specific to Xtu and Xtt

The sequences of cereal leaf streak (Xt-CLS) LAMP primers published by Langlois et al. (2017) were used to search the genome sequence of Xt4699 which was downloaded from NCBI database. The genomic region containing those primers was identified. The 3 kb sequences in that region were extracted and aligned to the corresponding regions of 35 sequenced *X. translucen* strains from different pathovars and other bacterial strains using Multalin Interface (Corpet 1988) to confirm the region that is specific to Xtt and Xtu. After the confirmation of region specificity, the two primers (F3/B3) from Xt-CLS LAMP primer set were directly used in my research: F3 (5'-AACGAGCGAAGCCGTATG-3') and B3 (5'-GCATCCAACTTGGCTACAGT-3'). The primer set was first tested for its specificity to Xtt and Xtu on the bacterial strains listed above using a conventional PCR. For comparison, the previous published T1/T2 primers: T1 (5'-AGTCGTAACAAGGTAAGCCG-3') and T2 (5'-CTATTGCCAAGGCATCCACC-3') (Maes et al. 1996) was also tested along with F3/B3.

Each PCR amplification was carried out with 1 µl of g. DNA (5 ng/ul), 2 µl of 10x Taq reaction buffer, 2µl of 10x dNTP, 1.2 µl of 25mM $MgCl_2$, 0.8 µl of 10 µmol/liter each primer, 0.2 µl of Taq DNA polymerase (Bulls eye, Inc.), and 12.0 µl of ddH_2O in a total of 20 µl volume under the following conditions: 95°C for 5 min and 34 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 1 min, and 72°C for 5 min.

Development of the quantitative PCR

The qPCR reactions were conducted using 1 µl of g. DNA or bacterial washer extracts, 5 µl of the 2x concentrated master mix of a SsoAdvanced universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.), 0.4 µl of 10 µmol/liter each F3 and B3 primer, and 3.2 µl of PCR-grade water in a total of 10 µl reaction volumes. PCR amplifications were run on a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc.). The qPCR running program consisted of 95°C for 5 min and 40 cycles of 95°C for 15s and 58°C for 45s followed by a melt curve from 65°C to 95°C in increments of 0.5°C. Three biological and three technical replications were performed for each sample. The PCR-grade water was used as non-template controls. Data analysis was done with CFX Manager version 3.1 software (Bio-Rad Laboratories, Inc.) which allowed the baseline threshold to be set for each target to calculate Cq value. If a standard curve is present, the CFX software will automatically calculate reaction efficiency as well as show amplification traces, melt peak data, a full table of quantification results, and standard curve information. The target specificity of qPCR in the genome was determined by using 5-10 ng of purified genomic DNA from a total of 23 strains of Xtu and Xtt.

To establish qPCR standard curve for quantification, the bacterial cell suspension at OD_{600}=1.0 was made from the freshly grown LB10 culture and 10-fold serial dilutions of the suspension were made. The original and serial dilutions were plated on WBA (see above) to

estimate the bacterial concentrations. The linear regression equation of standard curve was generated in Excel by plotting Cq values against the corresponding log10 of equivalent bacterial cells of Xtu/Xtt.

Plant materials and bacterial cell extraction

i) Bacterial extraction from seeds. Seeds of ten hard red spring wheat varieties were collected from North Dakota State University Variety Performance Trials conducted at a Grand Forks County location in 2019 and 2020. In both years, a natural epidemic of BLS occurred, and field observations suggest a higher level of BLS in 2019 than in 2020. The varieties used for bacterial extraction included SY-Rockford, MS-Barracuda, TCG-Heartland, SY-Ingmar, LCS-Cannon, CP3910, ND-Barlow, ND-Glenn, ND-VitPro, SD-Boost. Their disease scores were listed in the NDSU Hard Red Spring Wheat Variety Guide (Ransom et al. 2019, 2020). These varieties range in the levels of BLS resistance and acres they are grown on in North Dakota.

Several barley seed samples were also tested for the presence of the bacterium using qPCR. Those seeds were harvested from barley lines: TR-306, Genesis, BCN-3, BCN-10c, and BCN-31a which were grown in the field where BLS was present, or from two barley lines NDB112 and Pinnacle which were grown in greenhouse where no or very little BLS was observed.

For each genotype, the seed package of each variety was mixed well and 1g of seeds was weighed and then soaked in 2 mL 0.85% cold saline solution containing 0.02 % Tween 20 to extract bacterial cells. The mixtures were kept at room temperature in the laboratory for 20 minutes and were vortex vigorously every 5 min. A 200 µl of supernatants from bacterial washer extracts was heated at 95°C for 5 min in a PCR thermal cycler and a 1 µl of extraction solution was used as a template for a qPCR assay. The experiment was repeated three times. Statistical

analysis was conducted to measure the correlation between the qPCR results (CFU/g) and disease scores for ten varieties using a web-based Pearson Correlation Coefficient Calculator at the socscistatistics.com.

ii) Bacterial extraction from leaves. A total of 42 leaf samples were randomly collected in the NDSU research field plots where BLS was observed. Leaf samples were divided into five groups based on the greenness of leaves and amount of disease symptoms on the leaves. Group 1 was relatively green and had a great amount of water-soaking symptoms. Group 2 was relatively green but had less amounts of water-soaking symptoms compared to group 1. Group 3 was dried and become senescent. Group 4 was dry and picked up from the ground after field harvest. Group 5 was relatively green but had no obvious BLS symptoms. Each group consisted of eight individual leaves that were subjected for qPCR and the averages of the eight were presented. The wheat variety RB07 which was highly susceptible to BLS was inoculated with the bacterial strain LB10 in the greenhouse and the leaves with obvious water-soaking symptom were taken as a positive control. The water was used as non-DNA negative controls. To extract bacterial cells from dry leaf samples, the whole leaf was cut off into small pieces, which were soaked in 3 mL cold saline solution for 20 min and vortexed vigorously every 5 min. For green leaves, a leaf sample having obvious water-soaking region was cut off with 1 $inch^2$-long-piece and then, soaked in 1ml of cold saline solution. A 200 µl of the supernatant from bacterial washer extracts was heated at 95°C for 5 min in a PCR thermal cycler and a 1 µl was used as a template for qPCR assay.

Results

Confirmation of the genomic region specific to Xtt and Xtu and primer specificity

Sequence alignment of 3 kb region from 35 bacterial genomes confirmed that the whole genomic region harboring LAMP primers is only present in Xtu and Xtt strains, not in other *X. translucens* pathovars and unrelated bacterial species (Figure 1.1). Therefore, the primer F3/B3 from LAMP primer set was directly used in the qPCR development. In the primer specificity tests, PCR with T1/T2 primer amplified a 139 bp band from all strains belonging to *X. translucens*, while PCR with F3/B3 amplified a 210 bp band only from Xtu and Xtt strains, indicating F3/B3 is specific to the two pathovars (Figure 1.2). The results also confirmed that T1/T2 is specific to *X. translucens* pathovars (Figure 1.2).

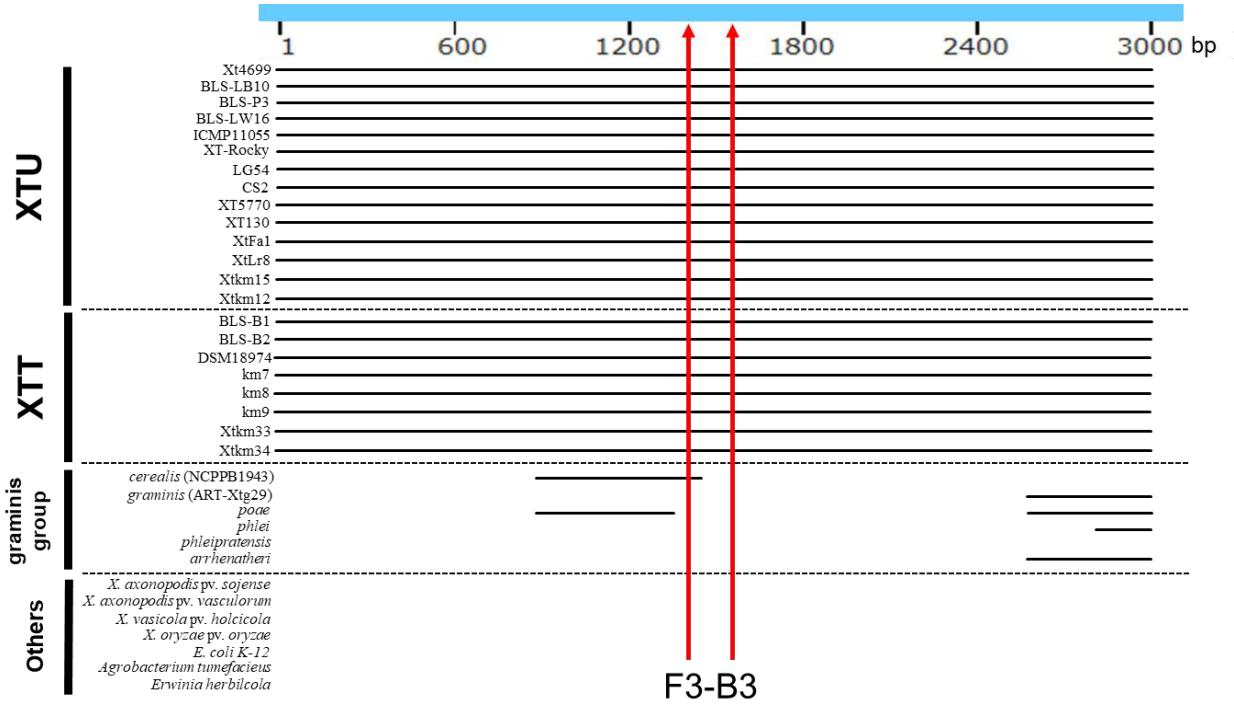


Figure 1.1. Sequence alignment to confirm the region that is specific to Xtt and Xtu. Sequence alignment was performed with 35 sequenced *X. translucens* and unrelated bacterial species using the 3 kb extended cereal pathovars-specific region sequences by Multalin Interface (Corpet 1988). 14 *Xanthomonas translucens* pv. *undulosa* (Xtu) and 8 *Xanthomonas translucens* pv. *tranclucens* (Xtt) strains, 6 *X. translucens* 'graminis group', and 7 unrelated bacterial species were used for this study.

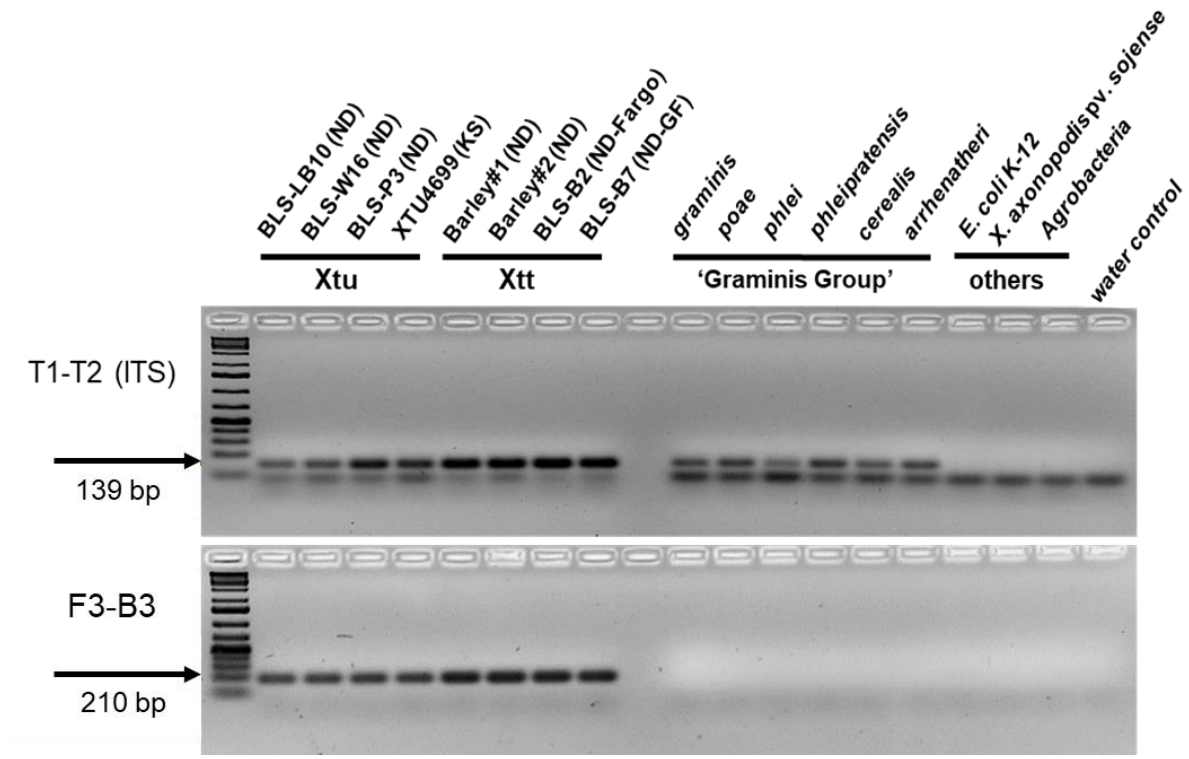


Figure 1.2. Agarose gel image of PCR amplification with two sets of primers in different bacterial strains. The bacterial strains included 4 *Xanthomonas translucens* pv. *undulosa* (Xtu) (BLS-LB10, LW16, P3, and Xt4699), 4 *Xanthomonas translucens* pv. *tranclucens* (Xtt) (Barley#1, Barley#2, BLS-B2, and BLS-B7), 1 strain each of *X. translucens* "graminis group" (*X. translucens* pvs. *graminis, poae, phlei, phleipratensis, cerealis,* and *arrhenatheri*), and 3 unrelated bacterial species (*E. coli* K-12, *X. axonopodis* pv. *sojense*, and *Agrobacterium tumerfaciens*). The primers were T1/T2 based on ITS region (Maes et al. 1996) and F3/B3 from cereal pathovars-specific LAMP primer set (Langlois et al. 2017). The 1 kb ladder (Thermo Fisher Scientific Inc.) was shown to the left and the band sizes for each amplicon were indicated.

Development of SYBR Green-based qPCR

The target specificity of F3/B3 primers was evaluated with in the melting curve analysis (Figure 1.3a). All samples showed positive amplifications with a melting temperature of 86.5°C with a single melting peak. This indicated that the primer has no unspecific target in the genome and qPCR assay is specific to Xtu and Xtt (Figure 1.3a).

Standard curve generated with 10-fold serially diluted bacterial cells was shown in Figure 1.3b. The mean Cq values for each dilution were 20.0 (1.2×10^9 CFU/ml), 22.1 (1.2×10^8 CFU/ml), 25.8 (1.2×10^7 CFU/ml), 29.4 (1.2×10^6 CFU/ml), 33.2 (1.2×10^5 CFU/ml), 36.7 (1.2

x 10^4 CFU/ml), and N/A (1.2 x 10^3 CFU/ml) and N/A (water control) (Figure 1.3b). A linear correlation between the mean Cq and log value of bacterial cell concentrations was observed with R^2=0.99, slope=-3.33, and PCR efficiency=99.6% (Figure 1.3b). The minimum detectable amounts of bacterial cells were shown ranging between 1.2x10^3 to 1.2x10^4 CFU/mL (Figure 1.3b).

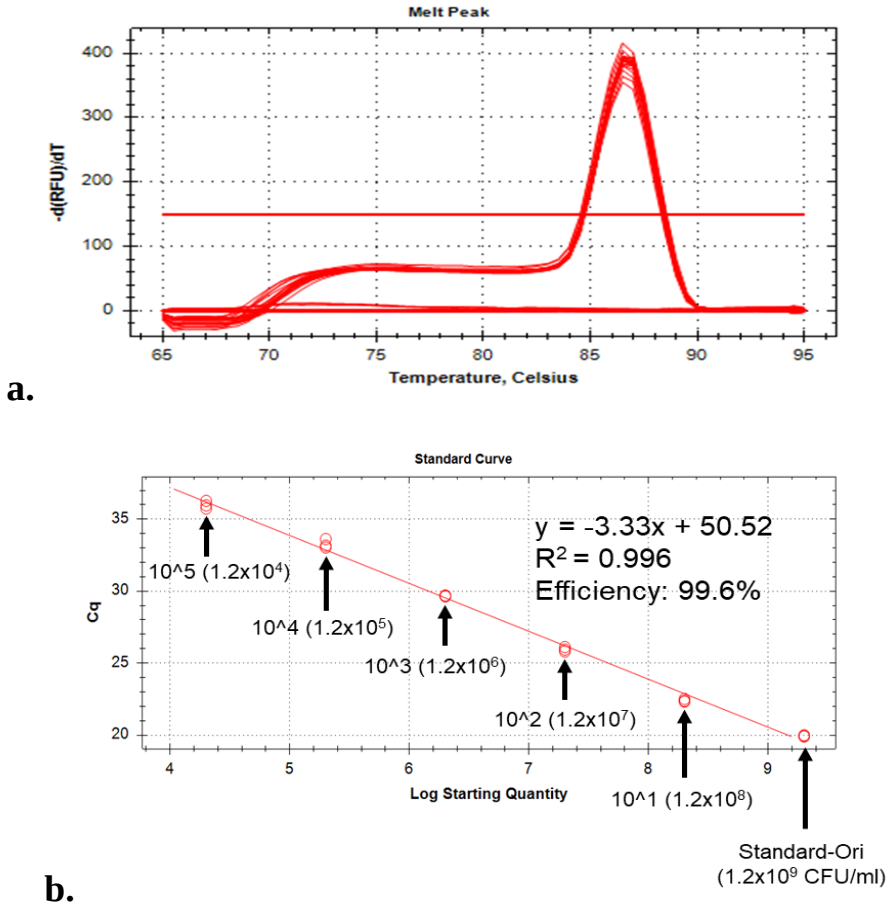
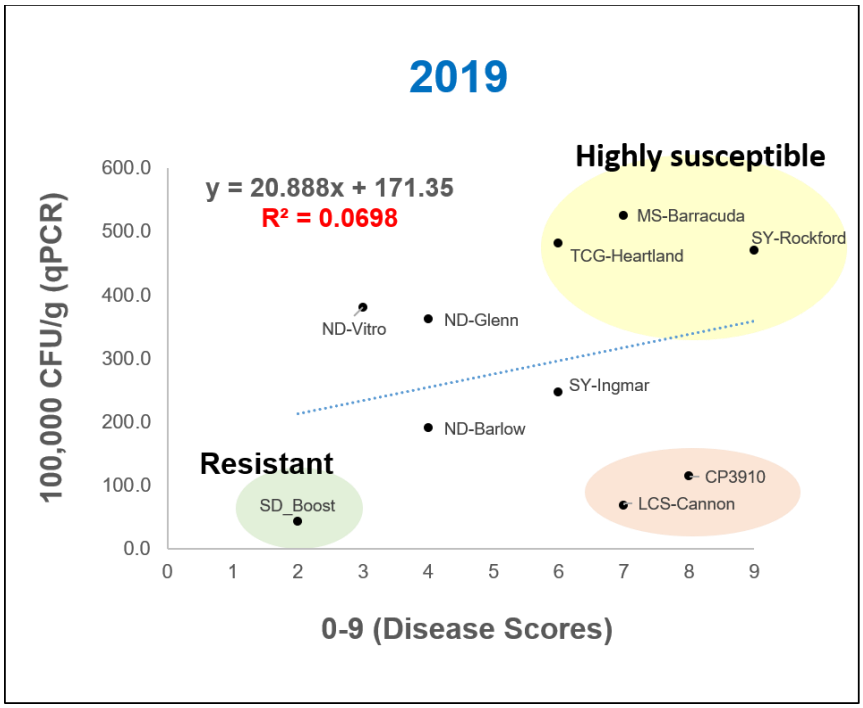


Figure 1.3. Target specificity confirmation and standard curve of the developed qPCR method. (a). SYBR Green-based melting curve analysis, demonstrating the specificity of the F3/B3 primers to detect pure culture bacterial cells of Xtu strain, BLS-LB10 with melting temperature at 86.5°C. (b.) Standard curve of the quantitative PCR (qPCR). The pure bacterial culture of BLS-LB10 was grown on WBA media and the original bacterial suspension was adjusted to the concentration at OD_{600} =1.0. The 10-fold serial dilutions of the original bacterial suspension were made to establish bacterial cell concentration standard curve. The dilutions were plated on WBA media to determine their bacterial cell concentrations through plating counting. The standard curve equation (-3.33x + 50.52) determined by CFX Manager V3.1 software (Bio-Rad Laboratories, Inc.) was displayed on the graph. The qPCR had coefficient of correction (R^2) at 0.996 and the PCR efficiency value at 99.6%.

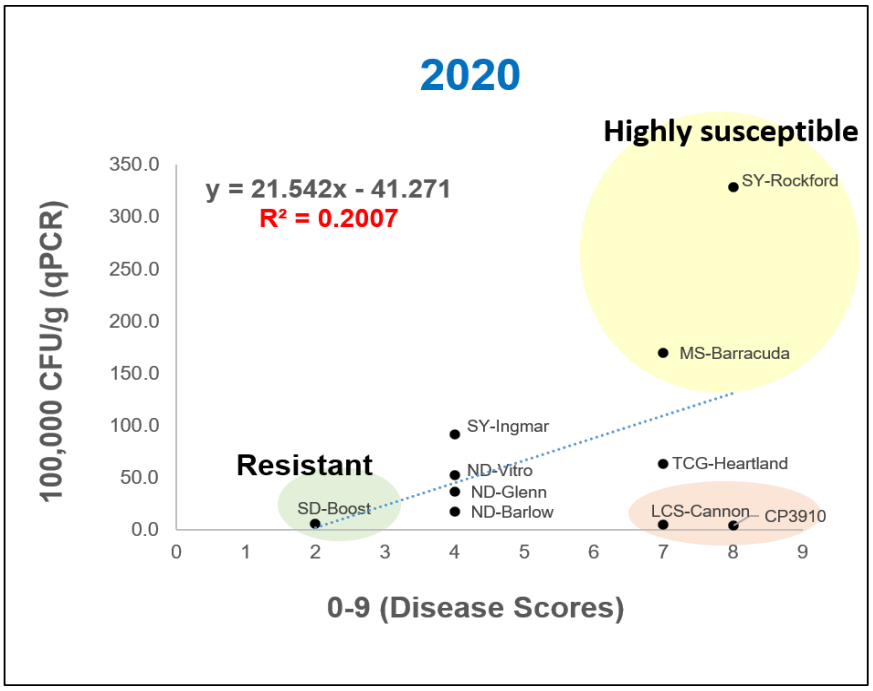
Quantification of bacterial cells in wheat seeds, leaves, and barley seeds

i) Wheat seeds The results of bacterial quantification in wheat seeds from 2019 and 2020 were shown in Figure 1.4a and 1.4b, respectively. The bacterial concentrations ranged from $4.1x10^6$ to $5.1x10^7$ for 2019 seeds (Figure 1.4a) and from $5.6x10^5$ to $3.3x10^7$ for 2020 seeds (Figure 1.4b). The bacterial concentrations were higher in 2019 than those in 2020, and align with field observations of BLS epidemics. Among the ten HRSW varieties, SD-Boost showed the lowest bacterial concentrations in both years while MS-Barracuda and SY-Rockford had the highest bacterial concentrations. This is correlated with our observations where SD-Boost was resistant and MS-Barracuda and SY-Rockford were highly susceptible. The two varieties LCS-Cannon and CP3910 had high disease scores but relatively low bacterial concentrations in both years, which was not correlated at all. The overall correlation coefficient (R^2) between bacterial concentrations (CFU/g) and disease scores for the ten HRSW varieties were 0.0698 in 2019 and 0.2007 in 2020 (Figure 1.4).

ii) Wheat leaves The positive control of (the inoculated RB07 leaves) had a concentration at $1.9\ x10^8$ CFU/g. The bacterial concentrations in different groups of leaves ranged from undetectable to $2.3x10^8$ CFU/g (Figure 1.5). The group 1 leaves which were largely green with high amounts of water-soaking symptoms had the highest concentration followed by that of Group 2 leaves where were largely green but had less amounts of disease symptoms. The dried leaves with slightly green area (Group 3) had very low number of bacterial numbers detected. There was no bacterial detection in the completely dried leaves fallen on ground (Group 4). A very low number of bacterial cells were detected in the green leaves without obvious disease symptoms (Group 5).



a.



b.

Figure 1.4. Dot plotting of bacterial cell concentration against disease scores for the ten hard red spring wheat (HRSW) varieties. (a). Data from 2019. (b). Data from 2020. X-axis indicates the disease scores of the ten HRSW varieties based on a 1-9 scale with 1 being resistant and 9 being highly susceptible. Y-axis indicates the bacterial concentrations in 100,000 CFU/g of ten HRSW varieties determined by qPCR. Linear regression equations were calculated using Excel and provided in each graph. R^2 indicates correlation coefficient calculated by a web-based Pearson Correlation Coefficient Calculator (Socscistatistics.com).

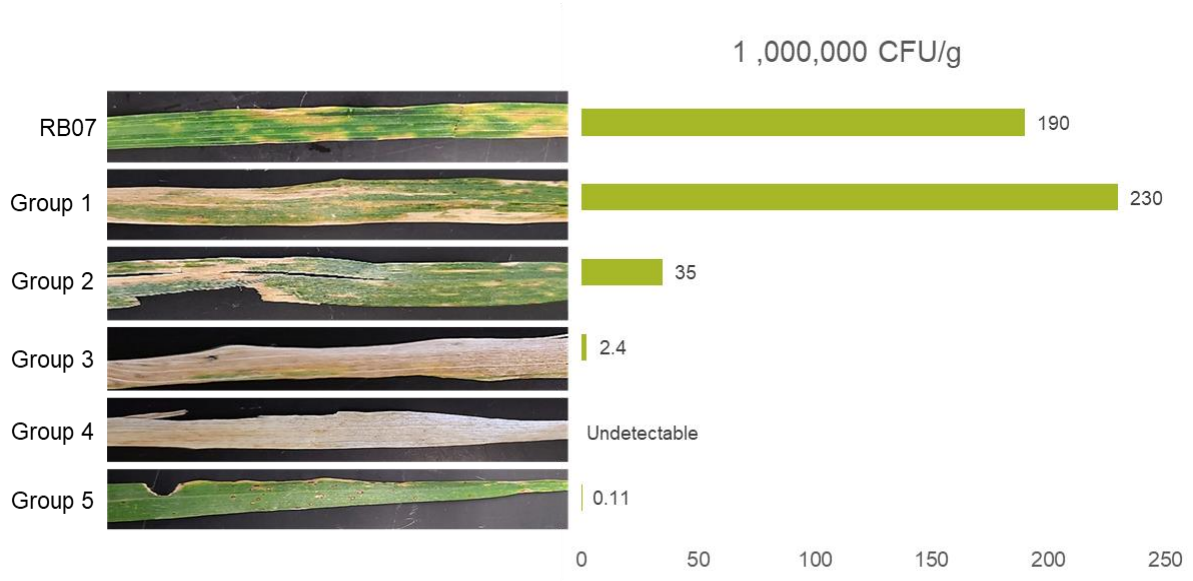


Figure 1.5. Bacterial quantification in different groups of leaf samples. The collected leaves were grouped based on greenness and number of water-soaking symptoms and the descriptions for each group were provided in Materials and Methods. The bacterial concentrations determined by qPCR were shown to the right of each leaf sample. The artificially inoculated leaves of RB07 were used as a positive control. Water was used as negative control in qPCR (data not shown).

iii) Barley seeds The qPCR detected bacterial cells in all barley seed samples harvested from field with TR306 having the highest bacterial concentration at $6.74x10^8$ CFU/g and BCN-31a having the lowest (Figure 1.6). However, the two seed samples (NDB112 and Genesis) harvested from greenhouse had no or very little bacterial numbers (Figure 1.6).

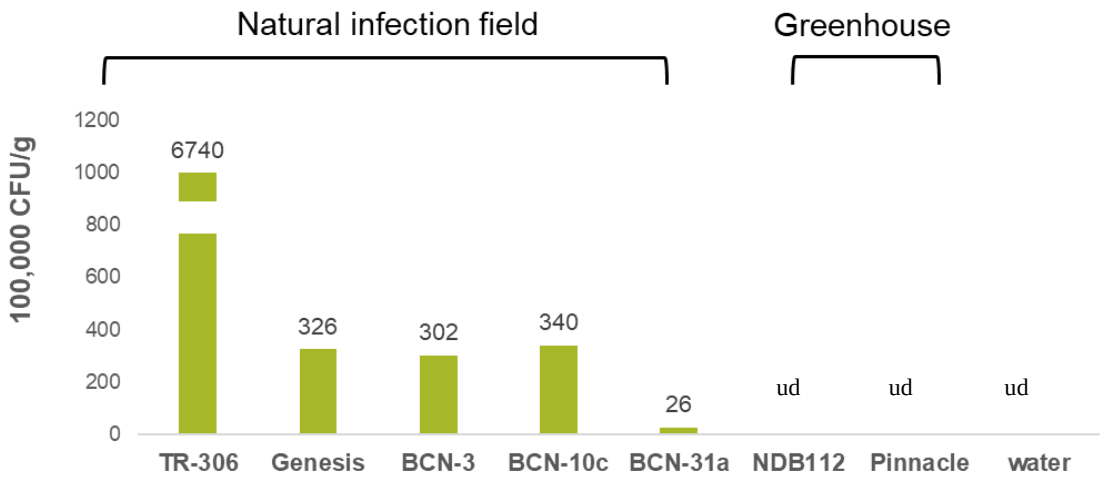


Figure 1.6. Bacterial quantifications in barley seeds. The seeds of two barley varieties (TR-306 and Genesis) and three germplasms (BCN-3, BCN-10c, BCN-31a) were collected from naturally infected fields. The seed samples of NDB112 and Pinnacle were harvested from green house. X-axis indicates different barley lines. Y-axis indicates bacterial concentration (100,000 CFU/g) in qPCR assay. Pure water was used as a negative control for qPCR. ud= undetectable.

Discussion

BLS has become major diseases on wheat and barley in the Upper Midwest regions including North Dakota. Currently, it is difficult to manage BLS largely due to the lacking of understanding on the overall biology of disease epidemiology. Although it is known that the bacteria can survive in infected seeds, we are not sure about how important role of the infected seed does play in the initiation of field epidemics. In addition, the importance of other source of primary inoculum, such as crop residues and grasses, is still undetermined (Duveiller et al. 1997). To provide more insight into disease epidemiology of BLS, a sensitive and reliable method to quantify the bacterial pathogens is needed. In this work, I established a qPCR method for specially detecting and quantifying Xtu and Xtt and further demonstrated that it can be used in quantifying the two bacteria in wheat and barley samples. As far as I know, this is the first report of a qPCR method for detecting and quantifying the two important bacterial pathogens.

The qPCR method would be a powerful tool in seed certification and disease epidemiology study.

Two diagnostic methods have been available for *X. translucens* before my study. One was developed by Maes et al. (1996), which is based on the unique sequence of the ITS region of *X. translucens*. The designed primer set T1/T2 could detect all *X. translucens* pathovars, but was unable to separate individual pathovars. The other diagnostic method was developed by Langlois et al. (2017) who identified genomic regions unique to each group or pathovars and used those regions to develop LAMP-based diagnostic method specific to each group. One set of LAMP primers was specific to cereal leaf streak (CLS) pathovars including Xtt and Xtu. Both T1/T2 PCR test and CLS-LAMP assay can be used to detect Xtu and Xtt in the infected seeds, but neither of them can be used for bacterial quantification. In addition, the conventional PCR test with T1/T2 has a low detection sensitivity and cannot separate Xtu/Xtt from other pathovars. LAMP assay has a better sensitivity and specificity and can be done on-site, but it often has a contamination issue and requires four primers in the assay to achieve higher specificity and sensitivity. The qPCR method I developed can detect as low as 1.2×10^4 CFU/ml of bacterial cells, which is similar to that of LAMP assay. However, in the study of bacterial disease epidemiology, sometimes a higher detection sensitivity is required. We could improve qPCR detection sensitivity through optimizing qPCR conditions or redesigning the primers as suggested by Tajadini et al. (2014).

The bacterial quantification on the ten HRSW varieties showed a sort of correlation between bacterial concentration and disease reaction in both 2019 and 2020 with 2020 being higher. Resistant cultivars tend to carry less number of bacterial cells and vice-verse. Milus and Mirlohi (1995) obtained a similar result when conducting plate counting assay of two winter

wheat varieties with different level of resistance. Boost had the lowest disease severity and carried lowest bacterial concentration in both years while a few varieties such as MS-Barracuda and SY-Rockford were highly susceptible also had a higher concentration. The two varieties CP3910 and LCS-Cannon consistently showed high disease score but relatively lower bacterial concentration in seeds, which made the correlation coefficient R^2 lower in both years. There could be a few possible reasons. First, we usually rate the disease based on the disease severity on the flag leaf, but seed samples harvested after the growth season were used for the qPCR assay. Therefore, there may not be a direct and simple connection between disease severity and seed bacterial concentration. Second, it is unknown whether bacteria can move directly from the flag leaves to the wheat heads and kernels. Third, it is possibly due to morphological characters of individual varieties, such as plant height, spike and seed morphology, which could make a difference in the bacterial infection on heads. More studies are needed to investigate the exact reasons for this discrepancy.

A wide variation in bacterial concentration was observed for barley seed samples. This is likely due to the fact that the seeds were from different sources. TR306 contained very high bacterial concentration in the seeds. It is likely that TR306 was highly susceptible to BLS and its seeds were harvested in a year when the disease pressure was extremely high. All the seeds from greenhouse had very little or undetectable amount of bacteria, which can be explained by little or no disease present under greenhouse conditions.

My research was the first to show that the pathogen Xtu can be detected and quantified in the wheat leaf samples by using qPCR. This is significant because we now can use this tool to make investigation into BLS disease epidemiology regarding the role of crop residues and weedy grasses playing in those disease epidemics. I detected high concentration of bacterial cells in

green leaves with large amount of BLS symptoms, but not much in the dry leaves, not at all in the dry leaves fallen on the ground. This may suggest that the bacterial pathogen reduce its population as leaves become old. This may also suggest that crop residues like dry leaves may not serve as a significant source of primary inoculum. However, no detection of the bacteria in dry leaves does not necessarily mean the absence of the bacterial pathogen in them. It is possible that the bacterial pathogen is present at the concentration that cannot be detected by this qPCR method due to the limitations of detection sensitivity (10^4 CFU/ml).

In conclusion, I established the first qPCR method for detecting and quantifying bacterial pathogens that cause important diseases on wheat and barley. This qPCR method will be useful in seed certification and provides a powerful tool for studying pathogen biology and BLS epidemiology.

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DEVELOPMENT OF DNA MARKERS TO DIFFERENTIATE TWO HIGHLY RELATED XANTHOMONAS TRANSLUCENS PATHOVARS: UNDULOSA AND TRANSLUCENS[2]

Abstract

Xanthomonas translucens contains a group of bacterial pathogens that are further divided into pathovars based on their host range. *X. t.* pv *undulosa* (Xtu) and *X. t.* pv. *translucens* (Xtt) are the two important pathovars that cause bacterial leaf streak diseases on wheat and barley, respectively (Adhikari et al., 2012; Kandel et al., 2012; Curland et al., 2018). The objective of this study was to develop DNA markers and a procedure to differentiate Xtu and Xtt and use it to characterize a collection of unknown *X. translucens* strains. DNA sequences unique to the two pathovars have been identified in Chapter 1. The SNPs within the sequences were examined to develop cleaved amplified polymorphism sequences (CAPS) markers for each pathovar. In addition, comparative genomics among the sequenced Xtt /Xtu strains revealed two Xtt unique DNA sequences that led to the development of two Xtt-specific markers, designated XopM-F/R and Xtt1-SP. I then used the newly developed markers to characterize a collection of 48 unknown *X. translucens* strains of different origins and also test the accuracy of those primers. Among them, 42 were identified to be Xtu, 4 to be Xtt, 1 to be other *X. translucens* pathovar and 1 to be non-*X. translucens*. The identity for those strains was confirmed by pathogenicity tests on wheat and barley plants indicating high levels of accuracy of those primers. The establishment of the efficient Xtt/Xtu quantification and differentiation methods will be powerful tools to study disease epidemiology and host pathogen interaction for two important bacterial pathogens.

[2] The material in this chapter was co-authored by Eunhye Hong, Dr. Zhaohui Liu, Dr. Rebecca Curland, and Dr. James Bucks. Eunhye Hong conducted the experiments and drafted this chapter. Dr. Curland and Dr. Bucks provided us genomic DNA strains for my experiments. Dr. Liu served as proofreader.

Introduction

BLS is a common disease on almost all wheat- and barley-growing regions in the world (Sapkota et al., 2020). In the US, BLS outbreaks have been sporadic and occurred mainly in Southern states where was relatively warm and humid (Bamberg, 1936; Duveiller et al., 1991). In recent years, BLS diseases of wheat and barley have been dramatically increased in the Upper Midwest of the US where weather conditions are typically cool and dry (Adhikari et al., 2012; Kandel et al., 2012; Curland et al., 2018; Sapkota et al., 2020). This region is major producers for hard red spring wheat and durum wheat, thus BLS has caused a great concern for wheat and barley productions in this region. Yield losses due to BLS have been reported to be 10% or less, but severe infections caused up to a 40% yield loss especially on highly susceptible varieties (Waldron, 1929; Forster et al., 1988; Duveiller et al., 1997). In addition, BLS affects the protein level leading to quality reduction in barley (Shane et al., 1987). Despite the fact that BLS has been one of major factors impacting wheat and barley production in many areas, controlling BLS in the field is nearly impossible due to lacking of effective management tools (Friskop and Liu, 2016). This is mainly because we do not have good understanding of the disease systems, including disease epidemiology, pathogen pathogenicity/virulence mechanisms, and host resistance/susceptibility as well as host pathogen interaction.

Bacterial leaf streak was first described on barley in 1917 (Jones et al., 1917), and on wheat in 1919 (Smith et al., 1919). The similar disease was later reported on other cereal crops and cereal-related grasses (Hagborg, 1942; Fang et al., 1950). It is now known that those diseases are caused by a group of highly related bacteria, collectively known as *X. translucens*, which can be further classified into different pathovars (Dye and Lelliott, 1974; Vauterin et al., 1995; Sapkota et al., 2020). The two pathovars causing disease on wheat and barley were named

as *X*. *translucens*. pv. *undulosa* (Xtu) and *X*. *translucens* pv. *translucens* (Xtt), respectively (Vauterin et al., 1995; Bragard et al., 1997).

Although Xtu and Xtt are not distinguishable morphologically and highly related, they differ in many aspects. First of all, the two pathovars have a distinctive difference in host range. Xtu is a broad host range pathogen capable of causing disease on a wide range of hosts, including wheat, triticale, barley, rye, and oat by artificial inoculations while Xtt has a narrow host range causing disease only on barley (Jones et al., 1917; Smith et al., 1919; Hagborg, 1942; Vauterin et al., 1995; Duveiller et al., 1997; Adhikari et al., 2012). Secondly, the modern phylogenetic studies using multilocus sequences and whole genome sequences have revealed that they are genetically separated belonging to two independent clades (Peng et al., 2016; Langlois et al., 2017; Curland et al., 2018, 2020). Thirdly, the two pathovars likely have specific tissue niches with Xtu mainly colonizing mesophyll tissues and Xtt being specialized in vascular tissue (Gluck-Thaler et al., 2020). Very recently, Gluck-Thaler et al. (2020) identified a hydrolase gene *CbsA* conferring vascular tissue specificity in Xtt. Fourthly, genome sequence comparison studies have shown that Xtu and Xtt have different sets of type III effector genes including transcriptional activator-like genes (TALEs) (Jaenicke et al., 2016; Peng et al., 2016; Charkhabi et al., 2017). Difference in effector repertoires suggests distinctive pathogenicity and virulence mechanisms for the two pathogens.

All those differences mentioned above strongly suggested the two pathovars cause two different diseases and should be separated for studying. Given the fact that the two pathovars can be isolated from the same hosts, a simple method to separate the two pathovars is needed. In this Chapter, my goal was to develop simple diagnostic markers for Xtu and Xtt. In previous Chapter, a genomic region was identified to be unique for Xtu and Xtt. I examined the single nucleotide

polymorphisms within that region between Xtu and Xtt to develop CAPS markers. In addition, I used the results from comparative genomics performed previously by our group to design Xtt-, or Xtu- specific markers.

Materials and methods

Bacterial strains

A total of 67 strains, including 44 Xtu and 23 Xtt were used in the development of Xtu or Xtt specific markers. The Xtu and Xtt strains were mainly collected from North Dakota (four different locations: Langdon, Lisbon, Casselton, Carrington) and Minnesota. ND strains were maintained in our lab and the MN strains were kindly provided by Dr. Rebecca Curland at the University of Minnesota. One Kansas strain was provided by Dr. Frank White at Kansas State University. The genomic DNAs were quantified using Nanodrop one microvolume UV-Vis spectrophotometers (Thermo Fisher Scientific) and adjusted to 5~6 ng/ul for PCR. Another set of 48 Xt strains with unknown identities were used in primer accuracy testing. Those strains isolated from different hosts and different geographic locations were used in this study (Table 1) and they were kindly provided by Dr. James Bucks from the University of Georgia. All bacterial strains were cultured on Wilbrink's Agar media (Bacto Peptone 0.5%, sucrose 1%, K_2HPO_4 0.05%, $MgSO_4.7H_2O$ 0.025%, Na_2SO_3 (anhydrous) 0.005%, and agar 1.5%) at 28 ºC for 2 days. The bacterial cells picked from a single colony were used in PCR for each strain. Those strains were tested with the newly developed primers (see below) as well as T1/T2 and F3/B3 primers (Chapter 1).

Table 1. Characterization of 48 unknown *Xanthomonas translucens* strains using genotyping and phenotyping.

Strains ID	Host	Location	Collection/Year	Pathogenicity Test (+ or -)		Identified pathovars by markers
				wheat	barley	
Xt-6	Wheat	Canada	NCPPB #1945	+	+	Xtu
Xt-13	Wheat	Canada	PDDCC #5755	+	+	Xtu
Xt-116	Wheat	South Dakota	1979	+	+	Xtu
Xt-118	Wheat	South Dakota	1979	+	+	Xtu
Xt-122	Wheat	Plains, GA	1982	+	+	Xtu
Xt-127	Wheat	Bozeman, Montana	1983	+	+	Xtu
Xt-131	Wheat	Ft. Valley, GA	1984	+	+	Xtu
Xt-133	Wheat	Griffin, GA	1991	+	+	Xtu
Xt-134	Wheat	Griffin, GA	1991	+	+	Xtu
Xt-214	Wheat	El Rufugio, Mexico	1978	+	+	Xtu
Xt-218	Wheat	Palotina, Brazil	1982	-	-	non-*X. translucens*
Xt-110	Triticale	Griffin, GA	1976	+	+	Xtu
Xt-111	Triticale	Tifton, GA	1978	+	+	Xtu
Xt-113	Triticale	Huntsville, AL	1978	+	+	Xtu
Xt-114	Triticale	Huntsville, AL	1978	+	+	Xtu
Xt-128	Triticale	Quincy, Florida	1984	+	+	Xtu
Xt-129	Triticale	Quincy, Florida	1984	+	+	Xtu
Xt-202	Triticale	Ciudad Obregon, Mexico	1978	+	+	Xtu
Xt-206	Triticale	Ciudad Obregon, Mexico	1978	+	+	Xtu
Xt-207	Triticale	Ciudad Obregon, Mexico	1978	+	+	Xtu
Xt-210	Triticale	Mexico CMI#B5562	1978	+	+	Xtu
Xt-211	Triticale	Celaya, Mexico	1978	+	+	Xtu
Xt-215	Triticale	El Batan, Mexico	1978	+	+	Xtu
Xt-216	Triticale	Toluca, Mexico	1977	+	+	Xtu
Xt-217	Triticale	Toluca, Mexico	1977	+	+	Xtu
Xt-226	Triticale	Ethiopia CMI#B6106	1978	+	+	Xtu
Xt-2	Rye	US	NCPPB #1836	+	+	Xtu
Xt-12	Rye	Canada	PDDCC #5749	+	+	Xtu
Xt-102	Rye	Tifton, GA	1977	+	+	Xtu
Xt-103	Rye	Tifton, GA	1977	+	+	Xtu
Xt-104	Rye	Ft. Valley, GA	1977	+	+	Xtu
Xt-105	Rye	Griffin, GA	1977	+	+	Xtu
Xt-108	Rye	Griffin, GA	1976	+	+	Xtu
Xt-109	Rye	Griffin, GA	1976	+	+	Xtu
Xt-112	Rye	Griffin, GA	1979	+	+	Xtu
Xt-115	Rye	Griffin, GA	1978	+	+	Xtu
Xt-125	Rye	Ft. Valley, GA	1980	+	+	Xtu
Xt-126	Rye	Athens, GA	1980	+	+	Xtu
Xt-130	Rye	Quincy, Florida	1984	+	+	Xtu
Xt-203	Rye	Ciudad obregon, Mexico	1978	+	+	Xtu
Xt-205	Rye	Ciudad obregon, Mexico	1978	+	+	Xtu
Xt-209	Rye	Ciudad obregon, Mexico	1978	+	+	Xtu
Xt-1	Barley	US	NCPPB #973	-	+	Xtt
Xt-3	Barley	US	NCPPB #1943	+	-	*X. translucens*
Xt-7	Barley	US	PDDCC #5752	-	+	Xtt
Xt-9	Barley	India	PDDCC #5735	-	+	Xtt
Xt-121	Barley	Montana	1981	+	+	Xtu
Xt-132	Barley	Quincy, Florida	1984	+	+	Xtu

+: pathogenic, -: non-pathogenic

CAPS marker development

To develop DNA markers for Xtu and Xtt differentiation, the genomic region unique to cereal pathovars (Langlois et al., 2017, see Chapter 1) was examined for SNP identification. The sequences of this region for sequenced Xtu and Xtt were downloaded from NCBI database and aligned to identify SNPs within the region using Multalin Interface (Corpet, 1988). The conserved SNPs were used to develop cleaved amplified polymorphism sequences (CAPS) markers. To develop CAPS marker, the restriction enzymes that cut specific SNP site were searched using a NEB Cutter 2.0 program (Vincze, 2003). The primers were designed to amplify the genomic region containing individual SNPs using web-based Primer 3.0 (Koressaar, 2018). The genomic region was amplified from testing strains using conventional PCR, which followed the same setup and the PCR program as described in Chapter 1. The PCR products were digested with the corresponding enzymes I identified. The digestion reaction was set up in 20 µl reaction consisting of 2µl of 10x a Cutsmart buffer with 1U of each restriction enzyme (New England Biolabs, Ipswich, MA) for 1h in a 37°C thermocycler. The digested products were separated on 2.0 % agarose gel.

Xtt or Xtu specific marker development

All available Xtu and Xtt genome sequences in NCBI GenBank as well as two unpublished Xtt genome sequences from our group were obtained. Bioinformatics tools and comparative genomics were done by our group to identify Type III secretion system effector genes unique to Xtt or Xtu (Liu et al., unpublished data). In addition, the genome region identified by (Sarkes et al., 2021), which was specific to Xtt and Xtu, was used to identify an adjacent region that is specific either for Xtt or Xtu. The primers for amplifying those specific T3

effector genes and specific genomic region were designed using web-based Primer 3.0 program (Koressaar, 2018).

Pathogenicity tests

To confirm the identities of Xt strains, pathogenicity tests were performed using wheat and barley plants. The two-row barley variety ND-pinnacle and the hard red spring wheat variety RB07 were used in pathogenicity test. The seeds of the two genotypes were sown in the cones with each cone containing two plants. The plants were grown in greenhouse room with temperature setting at 26~28°C till they reach three-leaf stage for testing. The bacterial strains were grown on WBA media (Chapter 1) for two days. The bacterial suspensions were made by scooping the bacterial culture into 1x PBS buffer (13.7mM NaCl, 0.27mM KCl, 1mM Na_2HPO_4, and 0.18mM KH_2PO_4). The concentrations of the suspension were measured for the OD value using BioSpectrometer (Eppendorf) and were adjusted to OD_{600} around 0.2. For inoculation, the bacterial solutions were infiltrated into leaf tissue using a syringe without a needle as described in Klement et al. (1990). The infiltrated plants were placed back in greenhouse room. The symptoms developed on the infiltrated area were recorded four days after infiltration. The development of water soaking indicated a pathogenic reaction and chlorosis development indicated nonpathogenic reactions. The experiment was repeated three times with each time having three biological replications.

Results

Marker developments

CAPS markers

Three SNPs, including A/C (Xtu/Xtt), C/A (Xtu/Xtt) and C/G (Xtu/Xtt) were identified within the region and each had a restriction enzyme to work with them, DdeI for A/C SNP,

BsaHI for C/A SNP, and BseYI for C/G SNP (Table 2). The information for the primers, the restriction enzymes and differential bands were shown in Table 2. The results of three CAPS markers were shown in Figure 2.1. CAPS marker I (DdeI-F/R) had two bands (135 bp and 273 bp) for Xtt strain B1, but had one band (408 bp) for Xtu strain LB10. The CAPS marker II (BsaHI-F/R) had one band (457 bp) or Xtt strain B1, but two bands (128 bp and 329 bp) for Xtu LB10. The CAPS marker III (BseYI-F/R) had two bands (55 bp and 263 bp) for Xtt but three bands (55 bp, 70 bp, and 193 bp) for Xtu.

Table 2. The primer sequence and other information for the three cleaved amplified polymorphism sequences (CAPS) markers.

CAPS marker	SNP	Primer sequences (5'-3')	Restriction enzyme	PCR product size (bp)
I: DdeI-F/R	Xtu: A Xtt: C	F-AAC ACA GGC AGC GTA TCG AC R-GAA AAG TTC GAT TCC GAC GA	DdeI	Xtu: 408 Xtt: 135+273
II: BsaHI-F/R	Xtu: C Xtt: A	F-GGC ATT TAT TCT GTG CTC CA R-CGA TCG GTA CCA CCA TCA G	BsaHI	Xtu: 128+329 Xtt: 457
III: BseYI-F/R	Xtu: C Xtt: G	F-TGT AGG TAC CCG AGA GTT GC R-CTA GCG ACA CTG CGC TTT TT	BseYI	Xtu: 55+70+193 Xtt: 55+263

F= Forward primer.
R= Reverse primer.
Xtu= *X. translucens* pv. *undulosa*,
Xtt= *X. translucens* pv. *translucens*

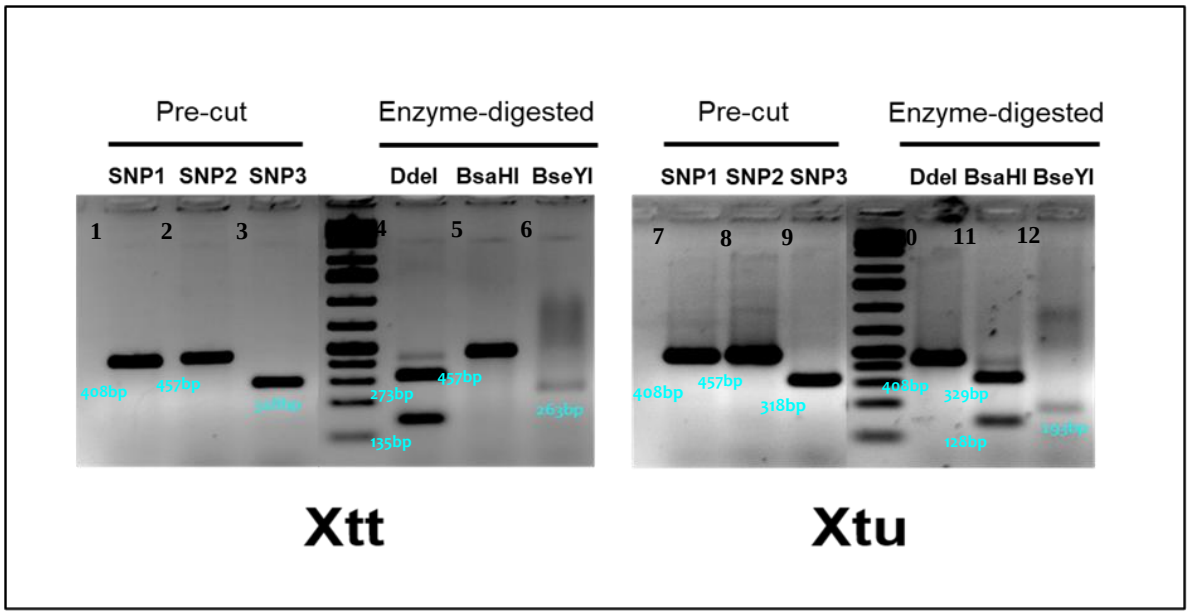

Figure 2.1. Agarose gel image of three CAPS markers on *X. translucen* pv. *translucens* (Xtt) and *X. translucens* pv. *undulosa* (Xtu). Lanes 1, 2, 3, 7, 8. 9: precut PCR products for three CAPS markers on the Xtt strain B1 and the Xtu strain LB10. Lane 4, 5, 6, 10, 11, 12: Enzyme-digested PCR products of three CAPS on the Xtt strain B1 and the Xtu strain LB10.

Xtt-specific primers

From comparative genomics, our group identified the T3SS effector XopM is present in all Xtt strains but not in all Xtu strains. Using the information from Sarkes et al. (2021), I identified a region that is also present only in Xtt strain, but absent in Xtu strain. Therefore, I designed primers for both sequences and tested on various Xtt and Xtu strains. PCR results showed that both pairs of primers amplified band only in Xtt strains (Barley#1, Barley#2, BLS-B2, and BLS-B7), but no band in Xtu strains (BLS-LB10, BLS-LW16, P3, and Xt4699) (Figure 2.2). The markers were designated as XopM and Xtt1. The primer information and the size of the markers were shown in Table 3.

Table 3. The primer information and size of two Xtt specific markers.

CAPS markers	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')	[a]Tem (°C)	PCR product Size (bp)
XopM-F/R	[b]F-AAC ACA GGC AGC GTA TCG AC	[c]R-GAA AAG TTC GAT TCC GAC GA	55°C	350
Xtt1-SP	[b]F-GGC ATT TAT TCT GTG CTC CA	[c]R-CGA TCG GTA CCA CCA TCA G	55°C	380

[a]Tem = Annealing temperature. [b]F=Forward primer, [c]R=Reverse primer.

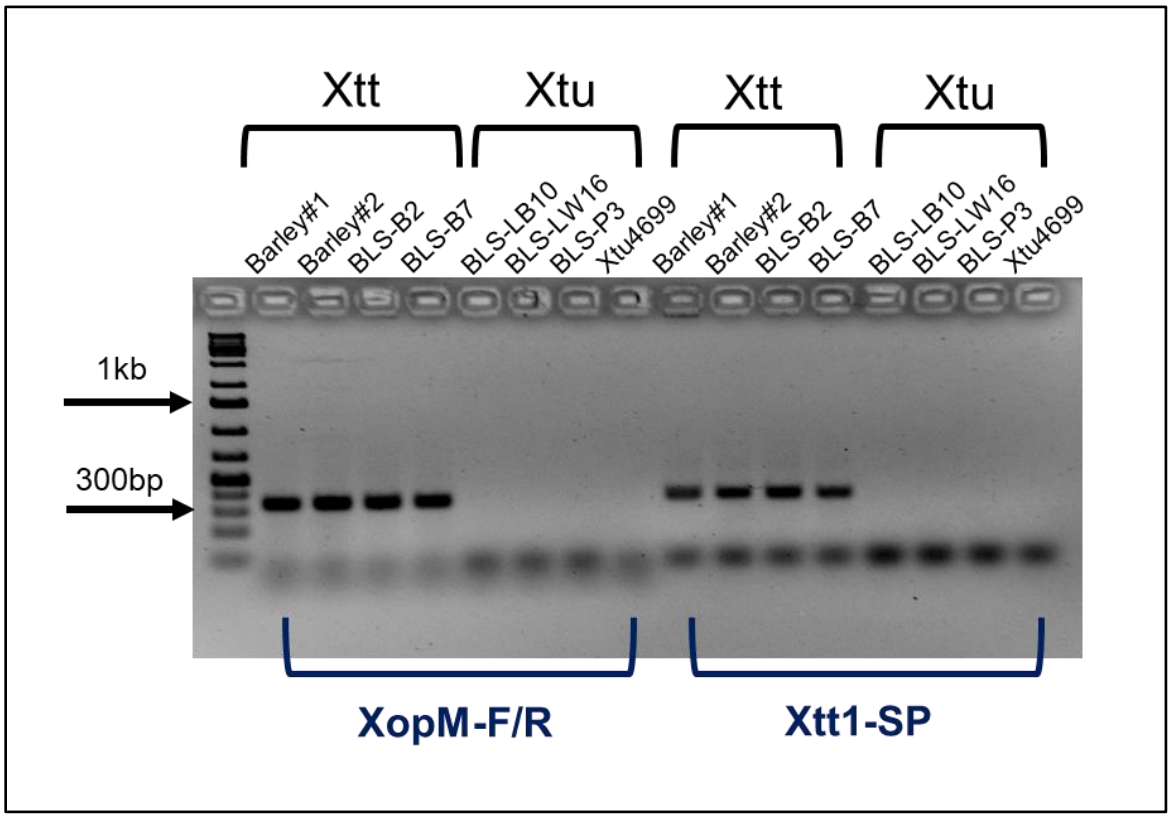

Figure 2.2. Agarose gel image of the two Xtt-specific markers. The markers were designated as XopM-F/R and Xtt1-SP, both of which are dominant in Xtt. Strains included Xtt strains: Barley#1, Barley#2, BLS-B2, BLS-B7 and Xtu strains BLS-LB10, BLS-LW16, bLS-P3, Xt4699. 1 Kb plus size standard was shown to the left with the 1 kb and 300 bp bands being indicated.

Testing of the markers on a set of known Xtu and Xtt strains

Two CAPS markers (DdeI-F/R and BsaHI-F/R) and two Xtt-specific markers (XopM-F/R and Xtt1-SP) were further tested on a set of Xtu and Xtt strains whose identities were previously known, including 40 Xtu and 18 Xtt strains. The two CAPS markers revealed only

one strain LG82 which did not match. LG82 was previously identified as Xtu, but it produced the Xtt type marker for the two CAPS markers (Figure 2.3). The XopM-F/R and Xtt1-SP revealed another strain LG100 besides LG82 that did not match (Figure 2.3).

To resolve the two mismatching, pathogenicity test was conducted for the two strains LG82 and LG100. The result showed that both LG82 and LG100 caused water-soaking symptoms on barley plants, but chlorosis (nonpathogenic) on wheat plants (Figure 2.4) indicating they are Xtt. As comparisons, the reference strains of Xtu (LB10) and Xtt (B1) and two randomly selected Xtu strains (CS37 and LB11) were included in the test and the results showed that their phenotypes matched to their genotypes (Figure 2.4).

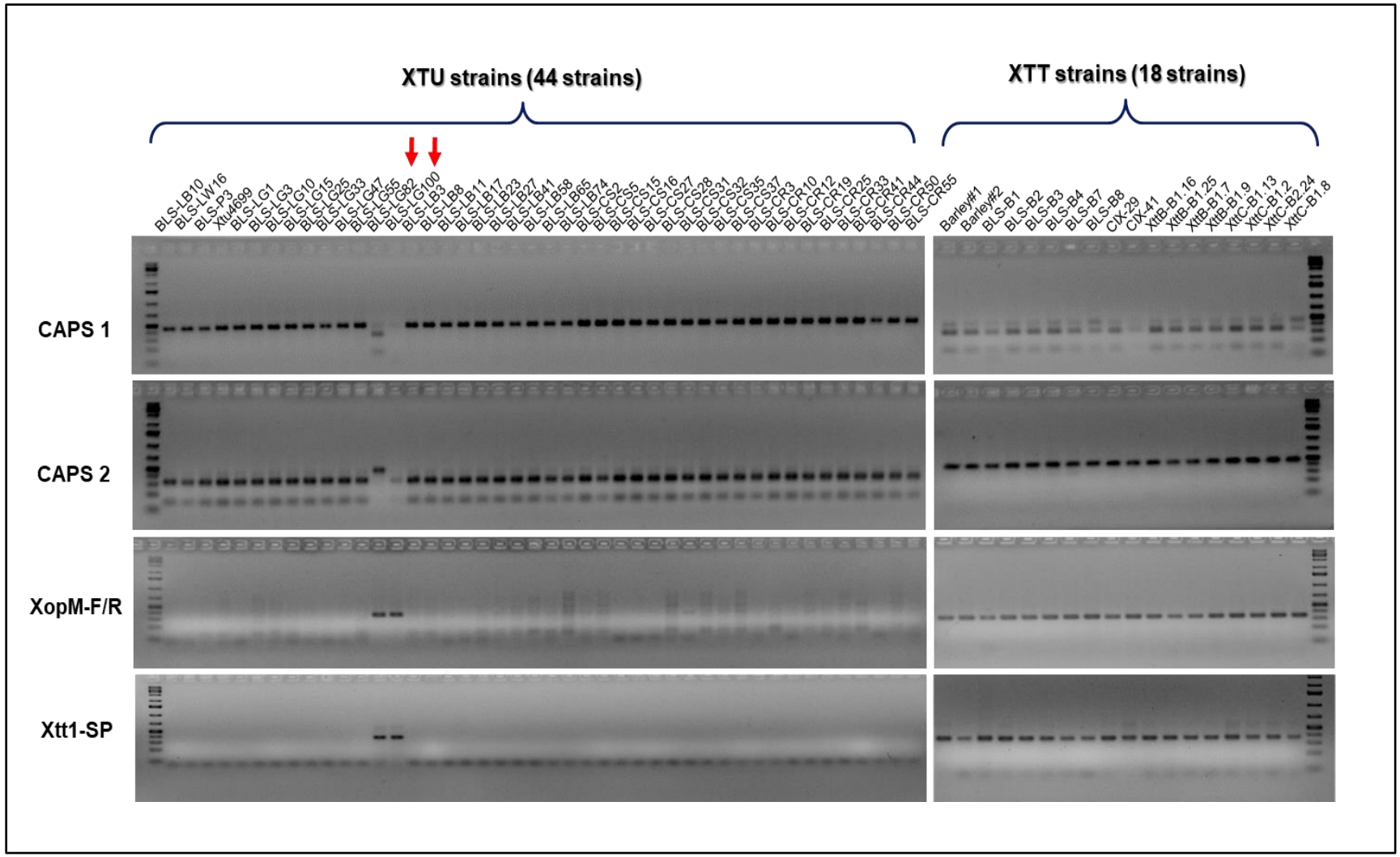

Figure 2.3. Agarose gel image of the two CAPS markers and two Xtt specific markers on set of known *Xanthomonas translucens* pv. *translucens* (Xtt) and *X. translucens* pv. *undulosa* (Xtu) strains. The strains included known 44 Xtu and 18 Xtt strains.

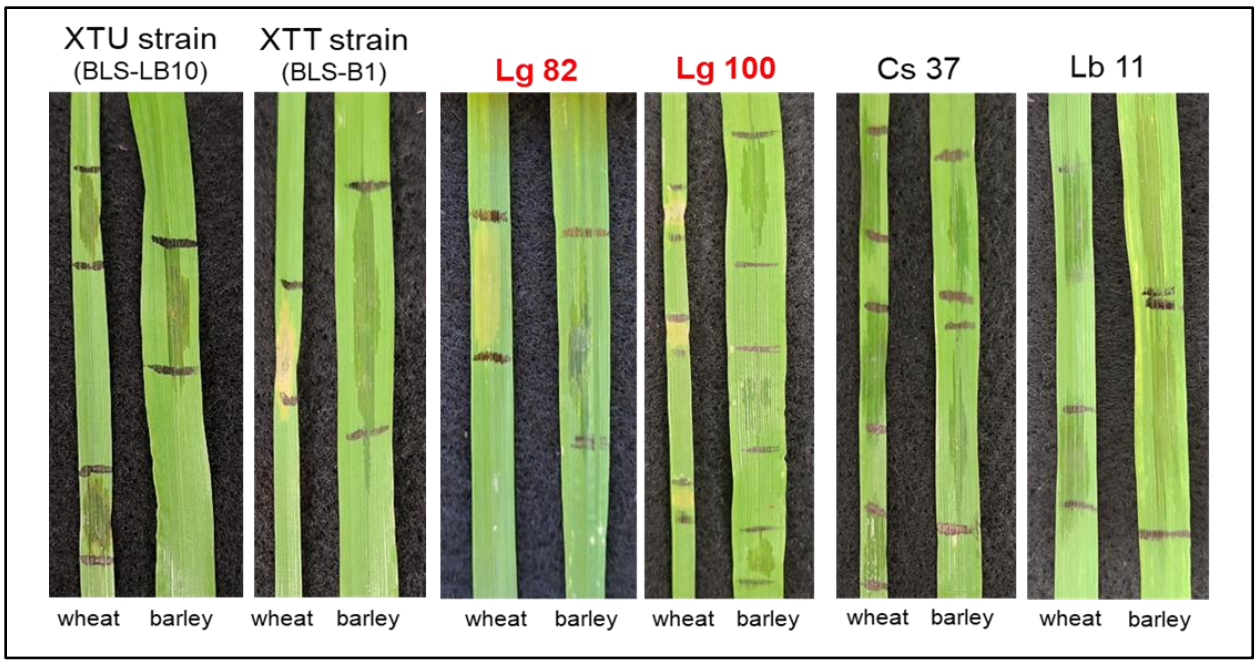

Figure 2.4. Photograph of pathogenicity tests of Xtu and Xtt strains on wheat and barley plants. The two strains: LG82 and LG100 were shown in the middle, the reference strains of Xtu (BLS-LB10) and Xtt (BLS-B1) were shown to the left and the two randomly selected Xtu strains (CS37 and LB11) were shown to the right.

Characterization of a set of unknown Xt strains using marker developed

By using T1/T2 primer, all the strains were confirmed to *X. translucens* except Xt-218 which had no band (Figure 2.5a). The F3/B3 primer was specific to Xtu and Xtt (Chapter 1). The results with primer pair F3/B3 revealed that Xt-3 was not belonging to the group of Xtu and Xtt because it did not produce a band (Figure 2.5b). By using the developed CAPS I marker and two Xtt-specific markers, four strains (Xt-127, Xt-1, Xt-7, Xt-9) were identified as Xtt and the remaining 42 strains were identified as Xtu (Figure 2.5 c, d, e).

All the 48 strains were phenotyped onto wheat and barley from pathogenicity test (Table 1). Four strains (Xt-127, Xt-1, Xt-7, Xt-9) identified as Xtt caused water-soaking symptoms on barley and chlorosis on wheat and all the 42 Xtu strains caused water-soaking symptoms on both wheat and barley (Table 1, Figure 2.6). The Xt-218 strain which was characterized to be non-*X. translucens* by genotyping caused no obvious symptoms on both wheat and barley (Figure 2.6).

Based on the genotyping results, Xt-3 was a *X. translucens* strain, but was neither Xtu nor Xtt. The strain produced a unique phenotype: strong chlorosis reactions on barley and weak water-soaking symptoms on wheat (Figure 2.6).

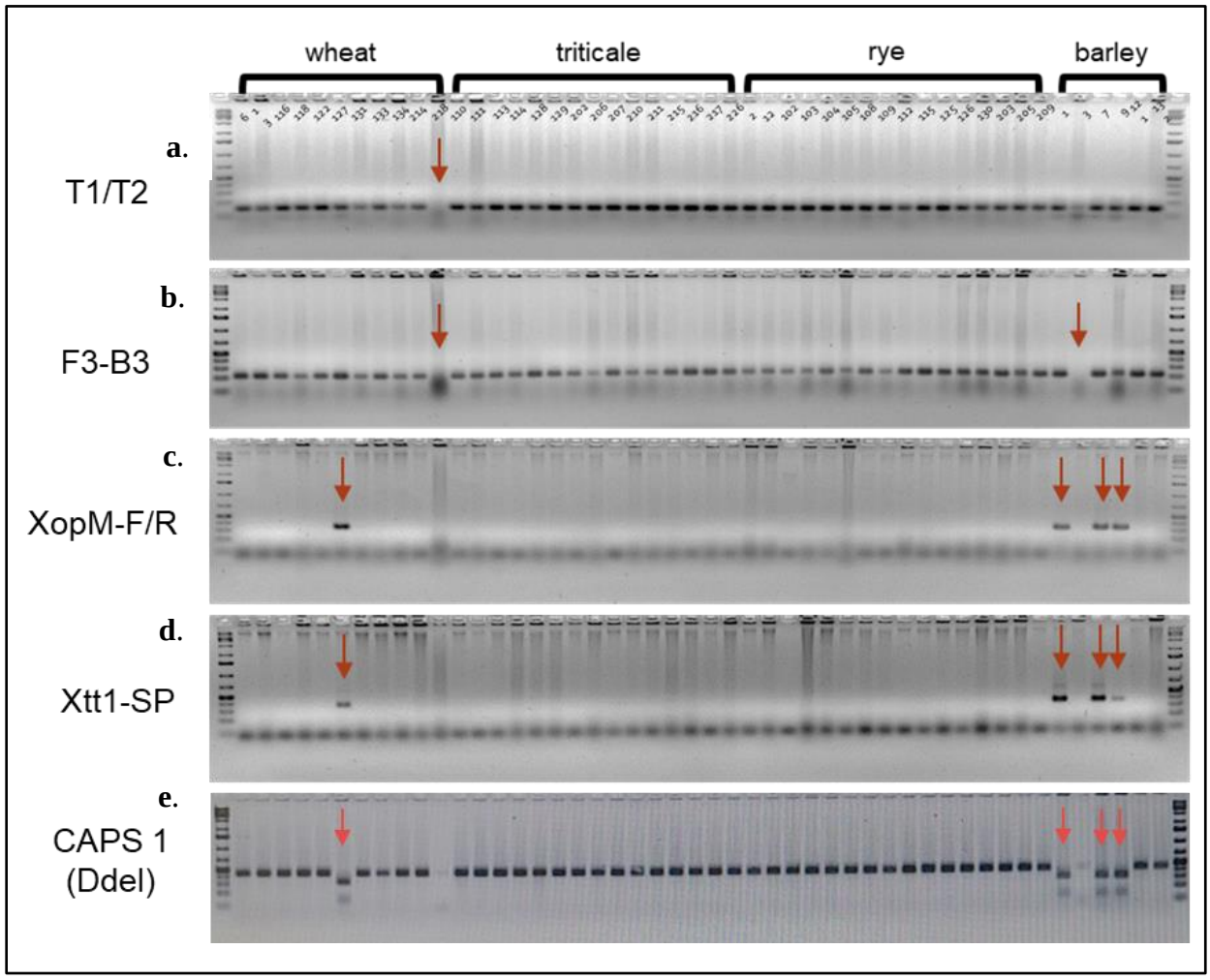

Figure 2.5. Agarose gel image of different markers on a set of unknown Xt strains. (a). marker run with ITS based primerT1/T2, (b). marker run with the primer F3/B3 specific to Xtu and Xtt, c. XopM-F/R, d. Xtt1-SP, and e. CAPS 1 marker 1 (DdeI-F/R). A total of 48 Xt strains were genotyped and they were isolated from wheat, triticale, rye, or barley). The red arrows indicate the strain (s) with a different marker type at individual marker locus.

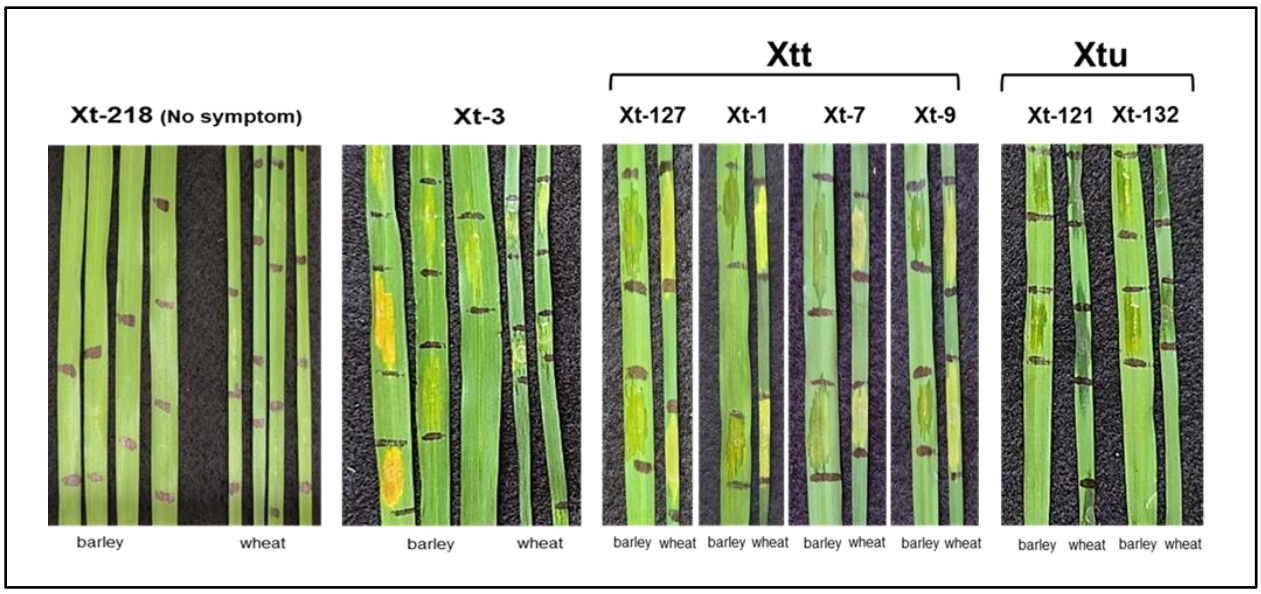

Figure 2.6. Photograph of pathogenicity tests of Xt strains on wheat and barley. Four Xtt strains (Xt-1, 7, 9, 127) and two Xtu strains (Xt-121, 132) were shown to the right. The Xt-218 strain (non-*X. translucens*), Xt-3 (not Xtt or Xtu) were shown to the left.

Discussion

Wheat and barley productions in North Dakota and elsewhere have been greatly impacted by BLS and not much is known for the two disease systems. The two pathovars that cause disease on wheat and barley are *Xanthomonas translucens* pv. *undulosa* (Xtu) and *X. translucens* pv. *translucens* (Xtt). The two pathovars differ in their host range and tissue specificity as well as pathogenicity or virulence mechanisms (Jones et al., 1917; Smith et al., 1919; Hagborg, 1942; Bragard, 1995; Vauterin et al., 1995; Duveiller et al., 1997; Adhikari et al., 2012; Gluck-Thaler et al., 2020, Sapkota et al., 2020), suggesting they should be separated to study. However, the two pathovars are morphologically indistinguishable and can be isolated from the same hosts. Therefore, A simple method to separate the two pathovars is needed. In this chapter study, I developed a few DNA markers that can be used to simply separate the two pathovars and successfully applied those markers to characterize a set of unknown Xtt and Xtu strains. This is the first report of molecular markers for Xtu and Xtt differentiation.

I also established a simple procedure with those markers and other previous published markers for characterizing and classifying Xtu and Xtt strains. The procedure contained 1) the first PCR testing with previously reported T1/T2 to determine the identity at the species level of *X. translucens*, 2) the second PCR testing with F3/B3 primers to identify Xtt and Xtu strains, and 3) the last PCR testing with my CAPS and Xtt specific markers to determine if they are Xtt or Xtu. Using this procedure, I successfully assigned to identity to a set of 48 bacterial strains with different host and geographic origins. The complete matching of phenotypic and genotypic data indicates the robustness of those markers and the procedure I developed.

I developed three CAPS markers and two Xtt specific markers, all of which are able to differentiate Xtu and Xtt. The three CAPS markers were developed from SNPs in the same genomic region. All three CAPS markers had the same accuracy on the strains I have tested Therefore, either of them should work. However, I suggest CAPS marker I or II to be used because they gave clear and robust bands (Figure 2.1). CAPS markers are co-dominant and are preferred to use, but they require two steps: PCR and restriction digestion. The two Xtt specific markers are dominant and require only a PCR step which was easier, but there could be a problem with false negative due to low DNA quality and quantity. It is very interesting to notice that LG100 strain (from North Dakota) was incorrectly characterized by CAPS markers, but correctly by two Xtt specific primers, suggesting that Xtt specific markers have a better accuracy than CAPS markers. Therefore, I suggest using both CAPS marker and Xtt specific markers for confirmation. It is also to be noted that my study only used a limited number of Xtu and Xtt strains, in particularly Xtt strains (a total of 24). The accuracy of these markers should be tested on more number and geographic diversity of Xtt and Xtt strains.

Xt-218 was identified to be non- *X. translucens*. This strain was isolated from wheat, but interestingly, it cannot cause the disease on wheat. In culture, the strain produced yellow colonies like other *X. translucens* strains, but the colonies were more mucous (data not shown). This strain could be an endophytic bacterium. We have consistently isolated many yellow colony bacteria from wheat leaves which cannot cause any disease on wheat and barley (Liu et al., unpublished data). Those strains cannot cause any symptoms on both wheat and barley, suggesting they may not be *Xanthomonas*. We can sequence its 16S DNA and determine its actual identity by NCBI BlastN search.

Xt-3 is a *X. translucens* strain, but not belonging to either Xtu or Xtt. From the symptoms observed, it is likely to be a *X. translucens* pv. *cerealis* (Xtc) strain. Xtc usually causes weak water-soaking and/or chlorosis on wide range of cereal hosts (Bragard et al., 1997). Several MLSA studies have suggested that Xtc strains are phylogenetically independent from Xtt and Xtu (Curland et al., 2018; Langlois et al., 2017; Peng et al., 2016). I only tested this strain on wheat and barley in this study. The future work could be to test its pathogenicity or virulence on other cereal crops and to do MLSA using the sequences of several house-keeping genes (Timilsina et al., 2015; Liu et al., 2017).

In summary, I developed several DNA markers that can be used to differentiate Xtu and Xtt and successfully applied those markers in characterization of a set of unknown *X. translucens* strains into Xtt and Xtu pathovars.

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APPENDIX: LIST OF BACTERIAL STRAINS USED IN THIS STUDY

Pathovars	Strains ID	Location*	Collection Year	Reference
X. translucens pv. *undulosa* (Xtu)	BLS-LG1	LG, ND	2009	Adhikari et al., 2012
	BLS-LG3			
	BLS-LG10			
	BLS-LG15			
	BLS-LG25			
	BLS-LG33			
	BLS-LG47			
	BLS-LG55			
	BLS-LG82			
	BLS-LG100			
	BLS-LB3	LB, ND	2009	Adhikari et al., 2012
	BLS-LB8			
	BLS-LB10			
	BLS-LB11			
	BLS-LB17			
	BLS-LB23			
	BLS-LB27			
	BLS-LB41			
	BLS-LB58			
	BLS-LB65			
	BLS-LB74			
	BLS-LW16	CS, ND	2008 and 2009	Adhikari et al., 2012
	BLS-CS2			
	BLS-CS5			
	BLS-CS15			
	BLS-CS16			
	BLS-CS27			
	BLS-CS28			
	BLS-CS31			
	BLS-CS32			
	BLS-CS35			
	BLS-CS37			
	BLS-CR3	CR, ND	2009	Adhikari et al., 2012
	BLS-CR10			
	BLS-CR12			
	BLS-CR19			
	BLS-CR25			
	BLS-CR33			
	BLS-CR41			
	BLS-CR44			
	BLS-CR50			
	BLS-CR55			
	BLS-P3	PS, ND	2009	Adhikari et al., 2012
	Xt4699	KS	1999	Peng et al., 2016
X. translucens pv. *translucens* (Xtt)	Barley #1	ND	2013	Peng et al., 2016
	Barley #2	ND	2013	Peng et al., 2016
	BLS-B1	Fargo, ND	2017	This study
	BLS-B2	Fargo, ND	2017	This study
	BLS-B3	Fargo, ND	2017	This study
	BLS-B4	Fargo, ND	2017	This study

	BLS-B7	GF, ND	2017	This study
	BLS-B8	GF, ND	2017	This study
	BLS-B9	GF, ND	2017	This study
	CIX-29	MN	2009	Curland et al., 2018
	CIX-41	MN	2009	Curland et al., 2018
	B1.11 (XttA)	AV, MN	2015	Curland et al., 2020
	B1.14 (XttA)	AV, MN		
	B2.1 (XttA)	KS, MN		
	B2.13 (XttA)	KS, MN		
	B1.16 (XttB)	AV, MN		
	B1.25 (XttB)	AV, MN		
	B1.7 (XttB)	AV, MN		
	B1.9 (XttB)	AV, MN		
	B1.13 (XttC)	AV, MN		
	B1.2 (XttC)	AV, MN		
	B1.8 (XttC)	AV, MN		
	B2.24 (XttC)	KS, MN		

* LB: Lisbon (ND), CS: Casselton (ND), PS: Prosper (ND), KS: Kansas, LG: Langdon (ND), GF: Grand Forks (ND), AV: Alvarado (MN), KS: Karlstad (MN)