Detection and differentiation of *Xanthomonas translucens* pv. *translucens* and pv. *undulosa* from wheat and barley by duplex quantitative PCR

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Abstract

Two probe-based quantitative PCR (qPCR) systems, namely P-Xtt and P-Xtu, were developed for diagnosis of cereal bacterial leaf streak pathogens *Xanthomonas translucens* pv. *translucens* and pv. *undulosa*, respectively. P-Xtt is specific to pv. *translucens*. P-Xtu is specific to pv. *undulosa*, pv. *cerealis*, pv. *secalis* and pv. *pistacia*. P-Xtt and P-Xtu worked on all accessible strains of pv. *translucens* and pv. *undulosa*, respectively. Both systems could detect 100 copies of the target gBlock DNA. The two systems could be used in both singleplex qPCR and duplex qPCR with similar efficiencies. On genomic DNA from strains of various *X. translucens* pathovars, both singleplex qPCR and duplex qPCR could specifically detect and differentiate pv. *translucens* and pv. *undulosa*. On infected barley and wheat grain samples, the duplex qPCR showed similar efficiency compared to a previously published qPCR system but with the additional capability of pathovar differentiation. The duplex qPCR system developed in this study will be useful in studies on bacterial leaf streak and detection/differentiation of the pathogens.

Keywords: bacterial leaf streak, Xtt, Xtu, pathovar *translucens*, diagnostics, qPCR

31 **Introduction**

32 *Xanthomonas translucens* is a Gram-negative bacterial pathogen causing bacterial leaf streak
33 (BLS) on a range of cereal crops, including wheat, barley, and rye (Jones et al. 1917). The
34 disease was firstly reported on barley (Jones et al. 1917) and wheat (Smith et al. 1919) in the
35 United States and the pathogens were named *Bacterium translucens* and *B. translucens var.*
36 *undulosum*, respectively. Once Dowson (1939) created the genus *Xanthomonas*, *B. translucens*
37 was reclassified as *X. translucens*. This pathogen is known to cause significant yield losses and
38 reduce crop quality (Bamberg 1936; Duveiller 1994), which can cause serious economic
39 implications for growers and food producers. According to Forster (1982), Idaho in the United
40 States has experienced yield losses of up to 40 percent. In Canada, Drayton (1923) firstly
41 reported that BLS on wheat and barley in Manitoba as early as 1922.
42
43 The taxonomy and nomenclature of *Xanthomonas* have undergone multiple changes due to new
44 studies on bacterial biology, phylogenetics, and genomics (Sapkota et al. 2020). The translucens
45 group was created to classify strains of *X. translucens* causing diseases on small grains (Bragard
46 et al. 1995; Vauterin et al. 1995). This group was believed to consist of three different biological
47 entities, pathovar (pv.) *translucens* pathogenic to barley, pv. *undulosa* pathogenic to both wheat
48 and barley, and pv. *cerealis* pathogenic to wheat, barley, oat and bromegrass (Bragard et al. 1995;
49 1997). Another pathovar, *X. translucens* pv. *secalis*, has been reported to be pathogenic to rye
50 (Reddy et al. 1924). However, phylogenetic studies indicated that strains of pv. *secalis* were
51 clustered with pv. *undulosa* (Bragard et al. 1997; Curland et al. 2018).
52

53 Timely and accurate detection and diagnosis of *X. translucens* are critical for the effective
54 management of this pathogen. The subspecies classification of *X. translucens* has presented
55 challenges in distinguishing between pathovars. While whole genome sequencing (Langlois et al.
56 2017) and multilocus sequence analysis (Curland et al. 2018; Ledman et al. 2021; Tambong et al.
57 2021) have been effective in identifying pathovars, they are time-consuming and impractical on
58 plant samples. One loop-mediated isothermal amplification (LAMP) protocol has been
59 developed (Langlois et al. 2017) for the specific detection of three *X. translucens* pathovars, but
60 cannot differentiate between them. A BLS-specific qPCR has been developed, but also cannot
61 differentiate between pv. *undulosa* and pv. *translucens* (Sarkes et al. 2022).
62
63 In recent years, there has been a rise in cases of BLS in wheat crops in the Midwestern United
64 States (Adhikari et al., 2012) and western Canada (Kuropatwa, 2014; Arnason, 2021; Tambong
65 et al., 2021), leading to an increased frequency of grain samples with BLS symptoms being
66 submitted to the Alberta Plant Health Lab (APHL) since 2019. The objective of this study was to
67 develop a qPCR-based diagnostic protocol that can detect, quantify and differentiate between
68 *Xanthomonas translucens* pathovars, specifically pv. *translucens* and pv. *undolusa*.
69
70 **Materials and Methods**
71 **Chemicals and standard techniques**
72 All chemicals and instrument were purchased from Fisher Scientific Canada (Ottawa, ON)
73 unless otherwise specified. All primers, probes and gBlocks were synthesized by Integrated
74 DNA Technologies (Coralville, IA). PCR was conducted in Promega PCR master mix with a
75 ProFlex PCR system. SYBR Green-based qPCR and probe-based qPCR were conducted in

76 SsoAdvanced universal SYBR Green supermix (Bio-Rad Canada) and PrimeTime gene
77 expression master mix (Integrated DNA Technologies), respectively, in a CFX96 touch real-time
78 PCR detection system (Bio-Rad Canada, Mississauga, ON).
79
80 **Bacterial isolates**
81 Isolation of X. *translucens* pv. *translucens* and pv. *undulosa* from wheat and/or barley grains
82 was conducted according to Sarkes et al. (2022). Two pv. *translucens* stains, namely CDCN-
83 B053 and B054, six pv. *undulosa* strains, namely CDCN-B045, B046, B047, B055, B057 and
84 B058, were isolated from barley and wheat leaf samples. The isolation and pathovar
85 identification were described in Sarkes et al (2022). Among these strains, CDCN-B046 and
86 CDCN-B053 were whole-genome sequenced (GenBank accession numbers JANVBQ010000026
87 and JANVBP010000007, respectively). Other bacterial species, if not otherwise specified, were
88 retrieved from the plant pathogen culture collection of APHL and their identities were confirmed
89 by sequencing of the 16S ribosomal RNA or the *cpn60* gene. PCR amplification of the 16S and
90 *cpn60* followed Klindworth et al. (2013) with the primer pair S-17/A-21 and Sahin et al. (2010)
91 with the primer pair H1594/H1595, respectively. Sequencing of the PCR products was done with
92 a SeqStudio genetic analyzer. All bacterial isolates were maintained on 2.3% (w/v) nutrient agar
93 in darkness at 28°C.
94
95 **DNA preparation**
96 Two 394-bp double-strand DNA fragments, with a sequence of GenBank accession number
97 LT604072 (nt: 4206041 to 4206434) and CP043500 (nt: 4106698 to 4107091), were synthesized
98 as gBlocks and named as G-Xtt and G-Xtu, respectively. Upon receiving, each gBlock was

99 dissolved in water and a stock solution at 8.3 pM was prepared, which roughly equals to 0.5 ×
100 10^7 molecules/µL.
101
102 All DNA extraction was conducted using the DNeasy Plant Pro kits (Qiagen Canada, Toronto,
103 ON) with a Qiacube (Qiagen Canada). DNA extracted from each sample was eluted in 50 µL
104 elution buffer. For DNA extraction from bacteria, 1 mL cell suspension containing 1 × 10^7 cells
105 was centrifuged at 5,000 rcf for 5 min and then the supernatant was removed to leave 100 µL
106 remaining to avoid disturbing the cell pellet. DNA was extracted from the resultant 100-µL
107 samples.
108
109 Genomic DNA samples of other *X. translucens* strains were kindly provided by researchers in
110 the United States. These included two pv. *translucens* strains from Dr. Zhaohui Liu, North
111 Dakota State University, Fargo, ND, and four pv. *translucens* strains, four pv. *undulosa* strains,
112 one pv. *graminis* strain, one pv. *arrhenatheri* strain, one pv. *poae* strain, one pv. *phlei* strain, one
113 pv. *phleipratensis* strain and two pv. *secalis* strains from Rebecca Curland, University of
114 Minnesota, St. Paul, MN. Upon receiving, a 20-30 µL subsample was transferred from each
115 DNA sample into a new tube and diluted with water to a final volume of 100 µL. The DNA
116 concentration in each subsample was measured using the NanoDrop 1000 with water as the
117 blanking sample.
118
119 **Primer design**
120 In a previous study (Sarkes et al. 2022), 27 genes from *X. translucens* pv. *translucens* strain
121 DSM18974 were selected as candidates for the development of the qPCR system specific to both

122 pv. *translucens* and pv. *undulosa*. Among these 27 genes, one gene (GenBank accession number:
123 SCB06379) was found present in pv. *translucens* only, i.e. not present in any other organisms
124 based on the current National Center for Biotechnology Information (NCBI) database
125 (https://www.ncbi.nlm.nih.gov). The genomic sequence of SCB06379 was retrieved and its
126 location on the whole genome sequence of DSM18974 (GenBank accession number: LT604072)
127 was identified. The sequence was extended to its 5' end and 3' end, both for 1,000 base pairs at
128 once, and then the extended sequence was used as the query to BLAST the *X. translucens* pv.
129 *undulosa* (taxid:487909) whole-genome shotgun contigs (wgs) database. The sequence extension
130 and BLAST were repeated until homologous sequences in the two pathovars were found. By this
131 approach, a heterologous region was identified (Fig. 1). Based on the sequence of SCB06379 and
132 the sequence of a pv. *undulosa* gene located in the heterologous region, two qPCR primer sets
133 were designed, respectively, using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-
134 blast). For each of the two primer sets, a probe was designed using Primer 3
135 (https://primer3.ut.ee). The probes in the two primer sets were labelled with different fluorescent
136 dyes (6-FAM for pv. *translucens* and HEX for pv. *undulosa*). The two primer/probe sets were
137 named P-Xtt and P-Xtu, respectively (Table 1).
138
139 **Primer sequence analysis**
140 All primers and probes were further analyzed by Blastn against the NCBI nr/nt database. For
141 each primer or probe, the presence of identical sequence (only one alignment, with 100%
142 coverage and 100% identity) in organisms other than *X. translucens* pathovars was checked.
143

144 The sequences of the primer target, i.e., the sequence from the 5' end of the forward primer site
145 to the 3' end of the reverse primer site, of the two qPCR systems were used as queries to Blast
146 two databases: nr/nt and wgs (Xanthomonas taxid: 338). The Blast results were manually
147 checked for the absence of sequences identical to the queries in species other than *X. translucens*
148 pathovars. In addition, the presence of P-Xtt and P-Xtu in all whole-genome sequenced *X.*
149 *translucens* pv. *translucens* and pv. *undulosa* was confirmed by checking the presence of all
150 whole-genome sequenced strains in the Blast results. The lists of whole-genome sequenced
151 strains of the two pathovars were obtained by searching "Xanthomonas translucens" in the NCBI
152 genome database (www.ncbi.nlm.nih.gov/genome) and manually checking the resultant Genome
153 Assembly and Annotation reports.
154
155 **qPCR reaction**
156 Each qPCR reaction was 20 µL containing 2 µL template and 0.25 µM of each primer. The
157 probe-based qPCR reaction also contained 0.15 µM probe. The qPCR program consisted of an
158 initial denaturation step at 95°C for 2 min, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C.
159 Each qPCR reaction was conducted with three technical repeats.
160
161 **Specificity test of P-Xtt and P-Xtu on the species level**
162 The specificity of the primer sets P-Xtt and P-Xtu was tested on templates from the following
163 organisms: *X. translucens* pv. *undulosa* strain CDCN-B046, pv. *translucens* strain CDCN-B053,
164 healthy wheat grains and healthy barley grains, pellets of 20 mL of YPG media (1% yeast extract,
165 1% peptone, 2% glucose, w/v) after incubating five healthy wheat grains and five healthy barley
166 grains at 150 rpm 30°C for 48 hours (the pellets were prepared by centrifuging the culture after

167 the grains being removed at 4,000 rcf for 5 min) and eight bacterial cultures on nutrient agar
168 including *Pseudomonas syringae* pv. *atrofaciens*, *P. syringae* pv. *syringae*, *Erwinia rhapontici*,
169 *Clavibacter michiganensis*, *Xanthomonas campestris* pv. *pelargonii*, *Pantoea agglomerans*, *P.*
170 *allii* and *P. ananatis*. Template DNA in each 20-µL reaction was 2 ng for bacterial samples or 10
171 ng for plant samples and the grain culture sample. All tests were conducted twice with similar
172 results.
173
174 **Specificity test of P-Xtt and P-Xtu on the pathovar level**
175 Genomic DNA from ten strains of *X. translucens* pv. *undulosa*, eight strains of pv. *translucens*,
176 and one strain of pv. *cerealis*, pv. *secalis,* pv. *graminis*, pv. *arrhenatheri*, pv. *poae*, pv. *phlei* and
177 pv. *phleipratensis* were tested by P-Xtu and P-Xtt in both singleplex and duplex qPCR. All
178 templates were also tested with the primer pair specific to *X. translucens* 16S region (F16S/R16S;
179 Sarkes et al. 2022) by SYBR Green qPCR, which served as DNA quantity control.
180
181 **Sensitivity test of P-Xtt and P-Xtu on gBlocks**
182 From the stock solution of each of the two gBlocks (G-Xtt and G-Xtu), a set of 10× serial
183 dilutions were prepared from 0.5×10^7 to 5 molecules/µL (seven solutions for each gBlock).
184 Using the serial dilutions as templates, sensitivity of P-Xtt and P-Xtu was evaluated in both
185 singleplex and singleplex qPCR reactions.
186
187 **Detection of *X. translucens* pv. *translucens* and pv. *undulosa* from plant samples**
188 Three barley grain samples and two wheat grain samples were tested by duplex qPCR using P-
189 Xtt and P-Xtu in the same reactions. The primers/probe set developed by Sarkes et al (2022) was

190 used as control. Preparation of samples for DNA extraction followed Sarkes et al. (2022). Briefly,
191 100 grains from each sample were soaked in 4 mL water in a 50-mL tube. The tube was vortexed
192 for 30 sec, from which a 1-mL sample of supernatant was transferred to a 1.5-mL tube. The tube
193 was centrifuged at 11,600 × *g* for 2 min and then 900 µL of the supernatant was removed. DNA
194 was extracted from the remaining 100 µL pellet/supernatant mixture, which represented all
195 extractible DNA from the surface of 25 grains.
196
197 **Data analysis**
198 In all qPCR, means of the quantification cycle (Cq) values from the three technical repeats were
199 calculated and treated as one data point. The qPCR standard curves were constructed by
200 regression analysis using the SAS software (version 9.4; SAS Institute, Cary, NC). qPCR primer
201 efficiencies were calculated as $E = -1+10^{(-1/slope)}$ (Svec et al. 2015). For the same template DNA
202 at the same concentration, the Cq values of singleplex qPCR and duplex qPCR were compared
203 by Student's t-test using the SAS software.
204
205 **Results and Discussion**
206 **Sequence analysis of the specificity of P-Xtt and P-Xtu**
207 Blastn of the primer and probe sequence against NCBI nr/nt database indicated that the forward
208 primer and the reverse primer of P-Xtt are present in one non-*X. translucens* organism,
209 respectively, and that the forward primer and the probe of P-Xtt are present in two and four non-
210 *X. translucens* organisms, respectively. However, all of these non-*X. translucens* are different
211 species. Aside from the above exceptions, all the primers and probes are present exclusively in *X.*
212 *translucens* pathovars. The results also indicated the specificity of P-Xtt and P-Xtu at the

213 pathovar levels, which are identical with the results from Blastn of the primer targets of P-Xtt
214 and P-Xtu as described below.
215
216 The primer targets of P-Xtt and P-Xtu are 103 and 93 bp, respectively (Table 1). Using the two
217 fragments as queries, Blastn against NCBI nr/nt database and wgs (Xanthomonas taxid: 338)
218 database was conducted (the results were verified on March 2, 2023; Table 2). For both
219 fragments, Blastn against the two databases covered all whole-genome sequenced *X. translucens*
220 pv. *translucens* and pv. *undulosa*, and did not produce any hit from a non-*X. translucens*
221 organism. Orthologs of the P-Xtt target was found in 23 out of the 27 whole-genome sequenced
222 pv. *translucens*, all the whole-genome sequenced pv. *graminis* (9), pv. *arrhenatheri* (3) and pv.
223 *hordei* (1), as well as seven unknown *X. translucens* pathovars. The P-Xtu target was found in all
224 the whole-genome sequenced pv. *undulosa* (18), pv. *cerealis* (3), pv. *secalis* (2) and pv. *pistacia*,
225 as well as 17 unknown *X. translucens* pathovars. Orthologs of the P-Xtu target are also present in
226 the four pv. *translucens* strains that lack the P-Xtt target: strains XtKm33, BLSB3, SIMT-07 and
227 SLV-2. On the target of the qPCR system F11/R11/P11 development by Sarkes et al. (2022),
228 these four strains had the same genotype as pv. *undulosa* rather than pv. *translucens* (Table S3 in
229 Sarkes et al. 2022). Phylogenetic studies (Khojasteh et al. 2019; Shah et al. 2021) indicated that
230 these four strains are indeed pv. *undulosa* but mislabeled as pv. *translucens*. Manually checking
231 the primer and probe sequences indicated that P-Xtt and P-Xtu are present in strains where an
232 ortholog of their target was present except for the forward primer of P-Xtt that was partially
233 present in the nine pv. *graminis* strains, the three pv. *arrhenatheri* strains and one unknown
234 pathovar. P-Xtt and P-Xtu, as well as their targets, are not present in pv. *poae*, pv. *phlei* and pv.
235 *phleipratensis.* These results indicated that P-Xtt is specific to pv. *translucens* and pv. *hordei*, P-

236 Xtu is specific to pv. *undulosa*, pv. *cerealis*, pv. *secalis* and pv. *pistacia*, and both P-Xtt and P-
237 Xtu are ubiquitous among strains of the specific pathovars. It has not escaped our notice that the
238 whole genome sequence of pv. *hordei* strain UPB458 was relabeled as pv. *translucens* (Table 2).
239 Thus, based on the database available genomic sequences, we can conclude that P-Xtt is specific
240 to pv. *translucens* only.
241
242 **Specificity of P-Xtt and P-Xtu on species level**
243 When P-Xtt and P-Xtu were tested on DNA from healthy (non-symptomatic) wheat and barley,
244 microorganisms associated with wheat/barley grain surface, and eight other bacterial species, no
245 positive signal was observed (Table 3). This result indicated that both P-Xtt and P-Xtu are
246 specific to *X. translucens* and DNA from host plants or other bacteria commonly associated with
247 wheat/barley grains will not interfere with the qPCR reactions.
248
249 **Specificity of P-Xtt and P-Xtu on bacterial DNA**
250 DNA from strains of *X. translucens* pathovars were tested by P-Xtt and P-Xtu by singleplex and
251 duplex qPCR (Table 4). Positive signals (mean Cq value less than 30) were produced by P-Xtt
252 from all pv. *translucens* strains but not from other pathovars. Cq values at 30 were produced
253 from the pv. *graminis* and pv. *arrhenatheri* strains, which in agreement with the sequence
254 analysis that the forward primer of P-Xtt is partially present in these two pathovars. Cq values
255 higher than 37 were produced from some strains of other pathovars, however, in most cases only
256 from one or two of the three technical repeats. Similarly, positive signals (mean Cq value less
257 than 30) were produced by P-Xtu from all pv. *undulosa*, pv. *cerealis* and pv. *secalis* strains but
258 not from other pathovars. Cq values higher than 37 were produced from some strains of other

259 pathovars, however, in most cases only from one or two of the three technical repeats. Both P-
260 Xtt and P-Xtu did not produce positive signals from pv. *poae*, pv. *phlei* and pv. *phleipratensis*.
261 These results confirmed that P-Xtt is specific to pv. *translucens*, P-Xtu is specific to pv.
262 *undulosa*, pv. *cerealis* and pv. *secalis*, and both P-Xtt and P-Xtu are ubiquitous among strains of
263 the specific pathovars.
264
265 Cq values generated by singleplex qPCR and duplex qPCR were compared by Student's t-test.
266 Among all the applicable samples, p values between 0.0813 and 0.9118 were obtained. These
267 results indicated that duplex qPCR with P-Xtt and P-Xtu in the same reaction had the same
268 robustness as singleplex qPCR on detection and differentiation of *X. translucens* pv. *translucens*
269 and pv. *undulosa*.
270
271 **Sensitivity of P-Xtt and P-Xtu on gBlocks**
272 The serial dilutions of the gBlock G-Xtt were tested in singleplex qPCR with P-Xtt and in duplex
273 qPCR with P-Xtt/P-Xtu. Similarly, G-Xtu was tested with P-Xtu and P-Xtt/P-Xtu. Based on the
274 Cq values, a standard curve was constructed for each primer set (Fig. 2). The efficiencies of P-
275 Xtt in singleplex and duplex qPCR are 0.98, respectively (Fig. 2a and 2b). The efficiencies of P-
276 Xtu in singleplex and duplex qPCR are 1.04 (Fig. 2c) and 1.03 (Fig. 2d), respectively. For P-Xtt,
277 on each concentration of the gBlock serial dilutions, the Cq value from singleplex qPCR (Fig. 2a)
278 and that from duplex qPCR (Fig. 2b) were compared by two-tailed Student's t-test. Of all gBlock
279 concentrations, the p values of the t-test were in the range of 0.1233 to 0.9095, indicating no
280 statistically significant difference in Cq between singleplex and multiplex reactions. Similar
281 results were observed for P-Xtu (Fig. 2c and d), in which the p values were in the range of

282 0.2416 to 0.9974. The results confirmed that P-Xtt and P-Xtu can be used in duplex qPCR and
283 singleplex qPCR with same robustness. In addition, all four standard curves (Fig. 2) indicated
284 that the low limit of the two qPCR systems for a positive detection in both singleplex and duplex
285 qPCR is 10 double stranded DNA copies per reaction. However, with consideration of the qPCR
286 results from bacterial genomic DNA in which a Cq value at 37 could be produced from non-
287 targeted strains (Table 4), and the influence of DNA extraction efficiency on diagnostic tests
288 (Yang et al. 2021), we suggest that the duplex qPCR system can reliably detect DNA from 100
289 bacterial cells.
290

291 **Detection of *X. translucens* pv. *translucens* and pv. *undulosa* from plant samples**

292 Both duplex qPCR using P-Xtt and P-Xtu and singleplex qPCR using F11/P11/R11 detected *X.*
293 *translucens* pv. *translucens* from the three barley samples and pv. *undulosa* from one of the two
294 wheat samples (Table 5). From the other wheat sample, no signal was detected, indicating the
295 grains in this sample were not infected by ether of the two pathovars. From the barley sample B2,
296 pv. *undulosa* was also detected by the duplex qPCR. This result was supported by bacterial
297 isolation: both pv. *translucens* and pv. *undulosa* were isolated from this sample and the identities
298 of the isolates were confirmed by sequencing of the 16S ribosomal RNA or the *cpn60* gene.
299 Our results indicated that P-Xtt and P-Xtu are specific to *Xanthomonas translucens* pv.
300 *translucens* and pv. *undulosa*, respectively. The two systems work for all the whole-genome
301 sequenced strains of these two pathovars and strains/DNA accessible to our lab. The two
302 primers/probe sets can be used in duplex qPCR to detect and differentiate the two pathovars.
303

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306

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379

380 **Table 1.** Primers and probes designed in this study
381

Primer set[a]		Sequence	GenBank accession number
P-Xtt	F	CGGAAGCCTGACGAGATGG	LT604072 Nucleotide: 4206294 to 4206396 (103 bp)[b]
	P	/56-FAM/AGAAATCCAGGGCCATCGTC/3IABkFQ/	
	R	TGCCTACGCCGGAATACCG	
P-Xtu	F	ACCACCTCGCCGCTCACAG	CP043500 Nucleotide: 4106793 to 4106885 (93 bp)[b]
	P	/5HEX/AAACGTCAT/ZEN/CGGCAGCATG /3IABkFQ/	
	R	CATGATGCTCGCCGATATGC	

382 [a]Primer sets for *Xanthomonas translucens* pv. *translucens* (P-Xtt) and pv. *undulosa* (P-Xtu), each consisting of a forward primer (F), a
383 probe (P) and a reverse primer (R).
384 [b]The fragment corresponding to the sequence from the 5' end of the forward primer to the 3' end of the reverse primer.

385 **Table 2.** Orthologs of the qPCR targets in the whole-genome sequenced strains of *Xanthomonas translucens* pathovars

Pathovar	Strain	GenBank number	Blast LT604072 (4206294-206396)[a]	Blast CP043500 (4106793-41068858)[b]	Database[c]
translucens	AB16-8-8a	JANVBV010000003	27384-27486	No Match	wgs
translucens	AB20-11-12a	JANVBS010000004	29978-30080	No Match	wgs
translucens	AB20-12-12b	JANVBR010000006	143557-143659	No Match	wgs
translucens	ATCC19319	JAJITG010000083	5099-5201	No Match	wgs
translucens	BC10-1-2a	JANUQZ010000003	143578-143680	No Match	wgs
translucens	BC10-1-2b	JANUQY010000006	143578-143680	No Match	wgs
translucens	BC11-3-3a	JANVCA010000012	89200-89302	No Match	wgs
translucens	BC13-4-5a	JANVBZ010000002	143578-143680	No Match	wgs
translucens	BC14-5-5b	JANVBY010000003	27384-27486	No Match	wgs
translucens	BC14-6-6a	JANVBX010000012	89200-89302	No Match	wgs
translucens	BC14-7-6b	JANVBW010000003	27384-27486	No Match	wgs
translucens	CDCN-B053	JANVBP010000007	143564-143666	No Match	wgs
translucens	CIX43	CP072988	3104098-3104200	No Match	nr/nt
translucens	CIX95	CP072990	3691826-3691928	No Match	nr/nt
translucens	DSM18974	CAPJ01000170	13120-13222	No Match	wgs
translucens	DSM18974	LT604072	4206294-4206396	No Match	nr/nt
translucens	Km7	CP064005	1169811-1169913	No Match	nr/nt
translucens	Km8	CP064004	3191872-3191974	No Match	nr/nt
translucens	Km9	CP064003	3740658-3740760	No Match	nr/nt
translucens	UPB458[d]	LWSV01000094	3822-3924	No Match	wgs
translucens	UPB787	JTEM01000253	6566-6668	No Match	wgs
translucens	UPB886	VCIH01000001	3836944-3837046	No Match	wgs
translucens	XtKm34	CP064001	980127-980229	No Match	nr/nt

386 [a,b]The targets of P-Xtt and P-Xtu were used as queries in Blast.
387 [c]Blastn was conducted against two database: the nr/nt database and the wgs database (Xanthomonas taxid: 338).
388 [d]Previously labeled as pv. *hordei*.

389 **Table 2.** Orthologs of the qPCR targets in the whole-genome sequenced strains of *Xanthomonas translucens* pathovars (Cont.)

Pathovar	Strain	GenBank number	Blast LT604072 (4206294-206396)[a]	Blast CP043500 (4106793-41068858)[b]	Database[c]
arrhenatheri	LMG727	CXOI01000063	1773-1875	No Match	wgs
arrhenatheri	LMG727	CP086333	4303903-4304005	No Match	nr/nt
arrhenatheri	UPB455	LWSX01000031	7756-7858	No Match	wgs
graminis	ART-Xtg10	FLTY01000287	2417-2519	No Match	wgs
graminis	ART-Xtg2	FLTV01000281	2190-2292	No Match	wgs
graminis	ART-Xtg29	FLTW01000291	4434-4536	No Match	wgs
graminis	ART-Xtg29	ANGG01000735	952-1054	No Match	wgs
graminis	ART-Xtg9	FLTU01000273	2401-2503	No Match	wgs
graminis	CFBP2053	LHSI01000001	3932637-3932739	No Match	wgs
graminis	ICMP6431	FLTZ01000271	2887-2989	No Match	wgs
graminis	LMG726	CP076254	4384134-4384236	No Match	nr/nt
graminis	UPB437	LWSW01000249	6664-6766	No Match	wgs
hordei	UPB458[e]	CP076249	451351-451453	No Match	nr/nt
unknown	B1	LNTA01000081	28705-28807	No Match	wgs
unknown	B1FA	CP090000	451895-451997	No Match	nr/nt
unknown	B2	LNSZ01000033	4132-4234	No Match	wgs
unknown	B8GF	CP089999	452595-452697	No Match	nr/nt
unknown	CFBP8304	CP074365	4126320-4126422	No Match	nr/nt
unknown	LB5[f]	LNSS01000139	50-152	No Match	wgs
unknown	XT8	LNVW01000003	4130-4232 (range 1)	No Match	wgs
unknown	XT8	LNVW01000003	292242-292344 (range 2)	No Match	wgs

390 [a,b]The targets of P-Xtt and P-Xtu were used as queries in Blast.
391 [c]Blastn was conducted against two database: the nr/nt database and the wgs database (Xanthomonas taxid: 338).
392 [e]Now labeled as pv. *translucens*.
393 [f]Carries the targets of both P-Xtt and P-Xtu.
394

395 **Table 2.** Orthologs of the qPCR targets in the whole-genome sequenced strains of *Xanthomonas translucens* pathovars (Cont.)

Pathovar	Strain	GenBank number	Blast LT604072 (4206294-206396)[a]	Blast CP043500 (4106793-41068858)[b]	Database[c]
translucens	BLSB3[g]	MADS01000080	No Match	14004-14096	wgs
translucens	SIMT-07[g]	MADQ01000160	No Match	3616-3708	wgs
translucens	SLV-2[g]	MADR01000243	No Match	2086-2178	wgs
translucens	XtKm33[g]	CP064002	No Match	1024529-1024621	nr/nt
undulosa	AB17-9-9a	JANVBU010000038	No Match	8493-8585	wgs
undulosa	AB17-10-9b	JANVBT010000037	No Match	4100-4192	wgs
undulosa	BLSW16	MADU01000070	No Match	13990-14082	wgs
undulosa	CDCN-B046	JANVBQ010000026	No Match	15106-15198	wgs
undulosa	CFBP2055	CP074361	No Match	4095104-4095196	nr/nt
undulosa	DOAB1058	JACONR010000071	No Match	8492-8584	wgs
undulosa	ICMP11055	CP009750	No Match	4242324-4242416	nr/nt
undulosa	LW16	CP043540	No Match	4207406-4207498	nr/nt
undulosa	MAI5034	CP089584	No Match	4114745-4114837	nr/nt
undulosa	NARK-1	MADT01000084	No Match	14051-14143	wgs
undulosa	P3	CP043500	No Match	4106793-4106885	nr/nt
undulosa	UPB513	MADV01000055	No Match	5857-5949	wgs
undulosa	XtFa1	CP063996	No Match	3656115-3656207	nr/nt
undulosa	XtKm12	CP064000	No Match	788227-788319	nr/nt
undulosa	XtKm15	CP063997	No Match	3621919-3622011	nr/nt
undulosa	XtLr8	CP063993	No Match	2897415-2897507	nr/nt
undulosa	Xtu4699	CP008714	No Match	4040196-4040288	nr/nt
undulosa	Xtu-UPB513	CP096570	No Match	1158486-1158578	nr/nt

396 [a,b]The targets of P-Xtt and P-Xtu were used as queries in Blast.
397 [c]Blastn was conducted against two database: the nr/nt database and the wgs database (Xanthomonas taxid: 338).
398 [e]Now labeled as pv. *translucens*.
399 [g]These four pv. *undulosa* strains were mislabeled as pv. *translucens* in NCBI database

400 **Table 2.** Orthologs of the qPCR targets in the whole-genome sequenced strains of *Xanthomonas translucens* pathovars (Cont.)

Pathovar	Strain	GenBank number	Blast LT604072 (4206294-206396)[a]	Blast CP043500 (4106793-41068858)[b]	Database[c]
cerealis	01	CP038228	No Match	3385846-3385938	nr/nt
cerealis	CFBP2541	JWHD01000016	No Match	1251724-1251816	wgs
cerealis	CFBP2541	CP074364	No Match	3999642-3999734	nr/nt
pistacia	ICMP16317	CP083804	No Match	3925920-3926012	nr/nt
secalis	CFBP2539	CP074363	No Match	4055150-4055242	nr/nt
secalis	NCPPB1943	MADM01000023	No Match	30140-30232	wgs
unknown	CR31	LNVJ01000003	No Match	131316-131408	wgs
unknown	CS2	LNVK01000002	No Match	8639-8731	wgs
unknown	CS22	LNVL01000002	No Match	119817-119909	wgs
unknown	CS4	LNVM01000050	No Match	15054-15146	wgs
unknown	DAR61454	AMXY01000020	No Match	31611-31703	wgs
unknown	LB10	LNVN01000002	No Match	8639-8731	wgs
unknown	LB5[h]	LNSS01000096	No Match	8639-8731	wgs
unknown	LG48	LNVO01000018	No Match	15298-15390	wgs
unknown	LG54	LNVP01000001	No Match	8639-8731	wgs
unknown	LW16	LNVQ01000045	No Match	9794-9886	wgs
unknown	P3	LNVR01000051	No Match	15300-15392	wgs
unknown	XT123	LNSU01000080	No Match	1138-1230	wgs
unknown	XT130	LNVS01000016	No Match	9273-9365	wgs
unknown	XT5523	LNVT01000017	No Match	90627-90719	wgs
unknown	XT5770	LNVU01000044	No Match	14016-14108	wgs
unknown	XT5791	LNVV01000001	No Match	5833-5925	wgs
unknown	XT-Rocky	LNST01000199	No Match	5872-5964	wgs

401 [a,b]The targets of P-Xtt and P-Xtu were used as queries in Blast.
402 [c]Blastn was conducted against two database: the nr/nt database and the wgs database (Xanthomonas taxid: 338).
403 [h]Carries the targets of both P-Xtt and P-Xtu.

404 **Table 3.** Specificity test of P-Xtt and P-Xtu on DNA of *Xanthomonas translucens* pv.
405 *translucens* and pv. *undulosa* and other organisms
406

	Cq value (mean±SD; n = 3)	
DNA template[a]	P-Xtt	P-Xtu
CDCN-B046 (*X. translucens* pv. *undulosa*)	ns	24.9±0.1
CDCN-B053 (*X. translucens* pv. *translucens*)	23.6±0.1	ns
Healthy wheat leaves	ns	ns
Healthy barley leaves	ns	ns
Healthy wheat grains	ns	ns
Health barley grains	ns	ns
Grain culture in YPG medium[b]	ns	ns
Pseudomonas syringae pv. *atrofaciens*	ns	ns
P. syringae pv. *syringae*	38.7[c]	ns
Erwinia rhapontici	ns	ns
Clavibacter michiganensis	ns	ns
Xanthomonas campestris pv. *pelargonii*	39.3[c]	ns
Pantoea agglomerans	ns	ns
P. allii	ns	ns
P. ananatis	ns	ns

407 [a]DNA template in a 20-µL qPCR reaction: bacteria, 2 ng; plants and the grain culture, 10 ng.
408 [b]DNA from microorganisms grown in YPG media (1% yeast extract, 1% peptone, 2% glucose,
409 w/v) after incubating healthy wheat and barley grains at 150 rpm 30°C for 48 hours
410 [c]Only one of the three technical repeats produced a Cq value
411

412 **Table 4.** Specificity test of P-Xtt and P-Xtu on DNA of *Xanthomonas translucens* pathovars by singleplex and duplex qPCR

Strain Name	Pathovar	Origin[a]	P-16S[b]	P-Xtt			P-Xtu		
				Singleplex	Duplex	t-test[e]	Singleplex	Duplex	t-test[e]
CDCN-B045	*undulosa*	APHL	17.1±0.2[c]	ns	ns	na	25.3±0.1	25.7±0.1	0.1005
CDCN-B046	*undulosa*	APHL	17.5±0.1	ns	ns	na	25.3±0.3	25.7±0.1	0.1480
CDCN-B047	*undulosa*	APHL	17.2±0.1	ns	ns	na	25.7±0.4	25.4±0.2	0.4324
CDCN-B055	*undulosa*	APHL	18.9±0.2	ns	39.4	na	27.1±0.1	27.2±0.1	0.5415
CDCN-B057	*undulosa*	APHL	19.1±0.1	38.1	38.4±1.0[d]	na	27.4±0.3	27.4±0.2	0.8439
CDCN-B058	*undulosa*	APHL	18.8±0.1	38.9	ns	na	26.8±0.4	26.6±0.1	0.6108
CIX23	*undulosa*	UM	17.8±0.2	ns	ns	na	26.4±0.2	26.6±0.2	0.4235
CIX40	*undulosa*	UM	17.9±0.2	38.4±1.3[d]	38.5±1.4[d]	0.9118	26.9±0.1	26.9±0.1	0.6842
CIX102	*undulosa*	UM	19.0±0.1	38.2±1.1[d]	38.4	na	27.7±0.2	27.8±0.2	0.4307
LMG892	*undulosa*	UM	18.8±0.1	38.9	ns	na	27.6±0.2	27.6±0.1	0.8463
CDCN-B053	*translucens*	APHL	18.0±0.1	23.4±0.4	23.3±0.3	0.8348	ns	ns	na
CDCN-B054	*translucens*	APHL	19.3±0.1	24.3±0.2	24.6±0.2	0.0977	ns	ns	na
XttB1FA	*translucens*	NDSU	15.9±0.1	21.3±0.2	21.3±0.3	0.8151	ns	ns	na
XttB8GF	*translucens*	NDSU	15.5±0.2	20.6±0.4	20.2±0.2	0.2720	ns	ns	na
CIX86	*translucens*	UM	16.1±0.1	22.2±0.1	22.2±0.2	0.6104	ns	37.9±0.2	na
CIX87	*translucens*	UM	18.2±0.1	24.2±0.2	24.2±0.2	0.8833	ns	ns	na
CIX94-95	*translucens*	UM	18.4±0.2	23.9±0.3	24.3±0.3	0.2170	ns	ns	na
LMG876	*translucens*	UM	16.4±0.1	22.0±0.3	22.5±0.3	0.1133	39.1	38.9±0.8[d]	na
LMG679	*cerealis*	UM	17.5±0.2	ns	38.6±1.7[d]	na	26.1±0.1	25.9±0.1	0.0813
LMG883	*secalis*	UM	16.7±0.1	39.1	38.4±0.6[d]	na	25.2±0.4	25.0±0.2	0.4993
LMG726	*graminis*	UM	18.6±0.2	30.3±0.2	30.4±0.5	0.6458	ns	39.1±0.4[d]	na
LMG727	*arrhenatheri*	UM	18.0±0.1	30.3±0.2	30.9±0.4	0.0939	39.2	38.8±0.6[d]	na
LMG728	*poae*	UM	17.6±0.1	37.0±0.2	37.4±0.9	0.4954	ns	ns	na
LMG730	*phlei*	UM	19.4±0.2	ns	38.7	na	ns	39.4	na
LMG843	*phleipratensis*	UM	17.1±0.1	37.3	ns	na	ns	38.6±0.3[d]	na

413 [a]Origin of strains or DNA. APHL, strains isolated by this study; UM, DNA provided by Rebecca Curland, University of Minnesota;
414 NDSU, DNA provided by Dr. Zhaohui Liu, North Dakota State University.
415 [b]Primer pair for the 16S region used in SYBR Green qPCR as DNA controls.
416 [c]Cq value shown as mean ± SD (n =3); ns = no signal from all of the three technical repeats; Data without a SD: only one of the three
417 technical repeat produced Cq.
418 [d]Two of the three technical repeat produced Cq.
419 [e]p value for two-tailed Student's t-test between the Cq value of the singleplex qPCR and that of the duplex qPCR; na = not applicable.
420
421

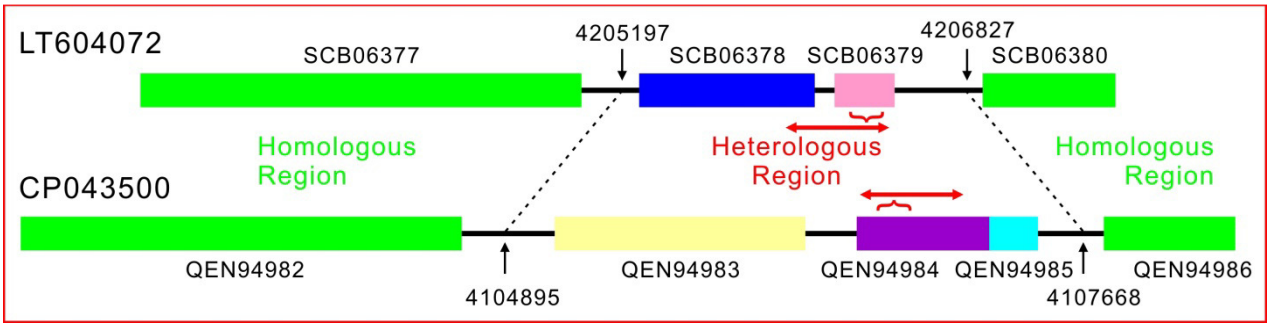
422 **Table 5.** Test of three barley grain samples (B1-B3) and two wheat grain samples (W1 and W2)

423 by duplex qPCR

Primers[a]	B1	B2	B3	W1	W2	CDCN-B046[b]	CDCN-B053[b]
P-Xtt	23.7±0.1[c]	25.6±0.1	21.6±0.2	38.1[d]	ns[e]	ns	22.8±0.1
P-Xtu	39.2[d]	25.9±0.1	ns	27.6±0.1	ns	25.3±0.1	ns
F11/P11/R11	24.5±0.1	25.0±0.1	22.5±0.1	26.8±0.2	ns	24.4±0.1	23.3±0.1

424 [a]P-Xtt and P-Xtu were used in duplex qPCR; the primers/probe set F11/P11/R11 (Sarkes et al.
425 2023) was used as control.
426 [b]The *X. translucens* pv. *undulosa* strain CDCN-B046 and the pv. *translucens* strain CDCN-B053
427 were used as control (2 ng DNA per qPCR reaction).
428 [c]Cq values shown as mean ± SD (n =3).
429 [d]Only one of the three technical repeat produced Cq.
430 [e]ns = no signal from all of the three technical repeats.

431 **Figures**



432

433 **Fig. 1.** Diagrammatic sketch illustrating the genomic positions of P-Xtt and P-Xtu. The
434 heterologous region, framed by homologous regions, on GenBank accession number LT604072
435 (*Xanthomonas translucens* pv. *translucens* strain DSM18974) and on GenBank accession
436 number CP043500 (*X. translucens* pv. *undulosa* strain P3) is indicated. The brackets indicate the
437 locations of P-Xtt and P-Xtu. The double-headed arrows indicate the location of the two gBlocks
438 (G-Xtt and G-Xtu).

439

440

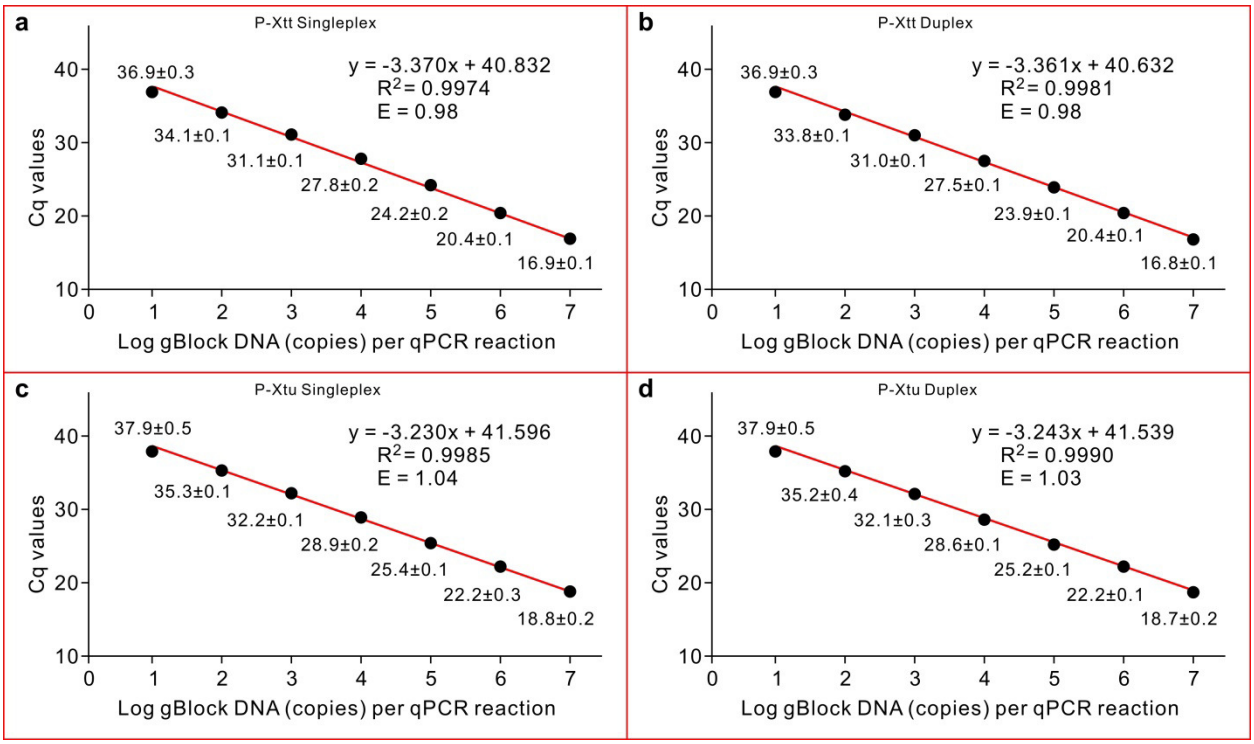


Fig. 2. Sensitivity of the qPCR systems P-Xtt for *Xanthomonas translucens* pv. *translucens* and P-Xtu for pv. *undulosa* on gBlock DNA. a and b, P-Xtt singleplex qPCR and P-Xtt/P-Xtu duplex qPCR on the pv. *translucens* gBlock, respectively. c and d, P-Xtu singleplex qPCR and P-Xtt/P-Xtu duplex qPCR on the pv. *undulosa* gBlock, respectively. The qPCR standard curves were generated from the mean of quantification cycle (Cq) values against log10 of gBlock DNA copies in one reaction. The R^2 score of the equation and the efficiency of the primers (E) are indicated over the curve. Efficiency was calculated as $E = -1+10^{(-1/slope)}$. Each data point is shown as mean ± SD (n=3).