Transcription Activator-Like Effectors Diversity in Iranian Strains of *Xanthomonas translucens*

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ABSTRACT

Bacterial leaf streak caused by different pathovars of *Xanthomonas translucens* is the most important seedborne bacterial disease of small grain cereals. However, variations in the virulence-associated genomic areas of the pathogen remain uninvestigated. In this study, the diversity of transcription activator-like effectors (TALE) was investigated using the Southern blotting of *Bam*HI-digested genomic DNAs in the Iranian strains of *X. translucens*. All 65 *X. translucens* strains were assigned into 13 genotypes, where 57 *X. translucens* pv. *undulosa* strains were placed in genotypes 1 to 8, and seven *X. translucens* pv. *translucens* strains were placed in genotypes 9 to 12. Interestingly, we did not find any TALE genes in the strain XtKm7 (genotype 13), which showed to be pathogenic only on barley. Virulence and aggressiveness of these strains in greenhouse conditions were in agreement with the TALE-based clustering

of the strains in the pathovar level, though variations were observed in the aggressiveness of *X. translucens* pv. *undulosa* strains. In general, strains containing higher numbers of putative TALE genes were more virulent on wheat and barley than strains containing fewer. This is the first TALE-based genetic diversity analysis on *X. translucens* strains and provides novel insights into the virulence repertoires and genomic characteristics of the pathogen. Further investigations using TALE mutagenesis and complementation analysis are warranted to precisely elucidate the role of each detected *X. translucens* TALE in bacterial virulence and aggressiveness either on wheat or barley.

Keywords: bacterial leaf streak, barley, diversity, TALE, wheat, *Xanthomonas translucens*

Bacterial leaf streak (BLS) caused by different pathovars of *Xanthomonas translucens* is the most important bacterial seedborne disease of small grain cereals, i.e., wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) around the world (Curland et al. 2018, 2020; Khojasteh et al. 2019; Langlois et al. 2017). Three distinct pathovars were identified within the *X. translucens* species as the causal agents of BLS based on the host range of the pathogen on different small grain cereals and wild gramineous weed species. *X. translucens* pv. *translucens* is mostly pathogenic on barley, although virulent strains on oat (*Avena sativa*), ryegrass (*Lolium* sp.), and Harding's grass (*Phalaris aquatic*) were also reported (Khojasteh et al. 2019). *X. translucens* pv. *cerealis* is pathogenic on bromus (*Bromus* sp.), oat, rye (*Secale* cereal), and wheat, while *X. translucens* pv. *undulosa* has a broader host range infecting barley, bromus, oat, rye, triticale, and wheat (Bragard et al. 1997;

Duveiller et al. 1997). Five additional pathovars, i.e., *X. translucens* pv. *arrhenatheri*, *X. translucens* pv. *graminis*, *X. translucens* pv. *phlei*, *X. translucens* pv. *phleipratensis*, and *X. translucens* pv. *poae*, were also reported to associate with different grasses with lower impact on the edible cereals (Maes et al. 1996; Vauterin et al. 1995).

Symptoms of BLS initially consist of linear water-soaked and translucent lesions on leaves, which expand under favorable environmental conditions and merge together turning into irregular glossy-surfaced stripes (Mehta 1990). Under high relative humidity and morning dew, bacterial exudates forming ooze droplets can be observed on leaf surfaces and blades (Mehta and Bassoi 1993). BLS is of high economic importance in irrigated fields, causing yield losses up to 40% by impairing grain fill and/or reducing kernel number (Duveiller et al. 1997; Schaad and Forster 1985). While the Iranian Plateau is considered the center origin of small grain cereals, geographic origin of the BLS pathogen is controversial. Recently, phylogeographic analyses on all available *X. translucens* strains worldwide using the sequences of four housekeeping genes suggested the Iranian Plateau as the center of diverse pathogenic strains (Khojasteh et al. 2019).

A number of phenotypic assays, molecular fingerprinting techniques, and phylogenetic approaches were used to evaluate diversity among *X. translucens* strains associated with BLS symptoms on small grain cereals. For instance, restriction fragment length polymorphism (RFLP); amplified fragment length polymorphism (AFLP); as well as BOX-conserved repetitive elements, enterobacterial repetitive intergenic consensus (ERIC), and repetitive sequence-based (REP) techniques were frequently used to elucidate population structure and genetic diversity of the pathogen (Adhikari et al. 2012; Bragard et al. 1995; Kölliker et al. 2006; Rademaker et al. 2005, 2006). These techniques were capable of discriminating among different lineages and pathovars of the BLS pathogen, which is in congruence with the host range of the strains and their phylogenetic position. However, none of the aforementioned approaches are associated with virulence repertoires and

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pathogenicity features of the pathogen, leaving most of the genomic properties of the *X. translucens* strains uninvestigated. Indeed, investigation of the genetic diversity of *X. translucens* using their virulence-associated genomic areas would help to predict the outcome of plant-microbe interactions in different lineages of the pathogen, while shedding light at the same time on the epidemiology and aggressiveness of various populations in different geographic areas (Ji et al. 2014; Ye et al. 2013).

Transcription activator-like effector (TALE) proteins play a pivotal role in the pathogenicity of plant pathogenic xanthomonads, contributing to in planta bacterial growth and symptom development (White and Jones 2018). TALEs share several common features including an N-terminal domain containing a type three secretion (T3S) signal, a C-terminal domain that harbors nuclear-localization signals (NLS), and an acidic activation domain (AD) specific to transcription factors (Mak et al. 2013). TALEs generally contain a series of tandem repeats of 33 to 35 polymorphic amino acids that confer DNA-binding specificity. Residues at the 12th and 13th positions are hypervariable and referred to as the repeat-variable diresidues (RVD) (Boch et al. 2009; Deng et al. 2012). TALEs are injected via the T3S system into host cells, where these proteins activate transcription of downstream genes for disease susceptibility or resistance by binding to the corresponding promoters (Kay and Bonas 2009; Kay et al. 2007; Römer et al. 2010; White et al. 2009). Different studies have demonstrated a strong and consistent association between the variations in TALE genes and bacterial virulence in different species of xanthomonads, i.e., *X. oryzae* pv. *oryzae* (Ji et al. 2016; Wu et al. 2007; Xu et al. 2019), *X. oryzae* pv. *oryzicola* (Ji et al. 2014) as well as *X. citri* subsp. *citri* (Ye et al. 2013), which we thought would be the case in the *X. translucens* strains associated with BLS of small grain cereals.

The main purposes of the present study were to (i) investigate TALE diversity in a collection of 65 *X. translucens* strains isolated from small grain cereals and weed species in Iran, and (ii) evaluate whether or not their virulence and aggressiveness on different host plants are associated with the Southern blot-based putative TALome of the strains. With this aim, genomic DNAs of 65 *X. translucens* strains isolated in eight cereal-growing provinces in Iran were subjected to Southern blot hybridization to determine the number and size of putative TALE genes, while the same strains were used for disease phenotyping (host range and aggressiveness) on barley and wheat plants.

MATERIALS AND METHODS

Bacterial strains. Sixty-five *X. translucens* strains used in this study were isolated from barley, wheat and gramineous weeds in the main cereal-producing areas in eight provinces in North-western, Western, Central, and Southern Iran from 2008 to 2017 (Table 1). Geographic distribution, host range on different gramineous species, and genetic diversity of the strains based on the multilocus sequence analyses and typing (MLSA/MLST) of four housekeeping genes (i.e., *dnaK*, *fyuA*, *gyrB*, and *rpoD*) were described previously (Khojasteh et al. 2019). According to the host range assays and MLSA scheme, 57 out of 65 strains were identified as *X. translucens* pv. *undulosa*, while eight strains were identified as *X. translucens* pv. *translucens*. All of the strains were restreaked onto nutrient agar (NA) and yeast extract-dextrose-calcium carbonate (YDC) agar media to confirm their morphological identity and Gram staining. The strains were resuspended in sterile distilled water and stored at 4°C for further use, while they were maintained in 15% glycerol at -70°C for the long-term storage.

Pathogenicity and aggressiveness assays. All of the *X. translucens* strains (Table 1) were evaluated for their pathogenicity and aggressiveness on barley ('Hua 30') and wheat ('Yangmai 158') plants (provided by Shanghai Academy of

Agricultural Sciences, China) under greenhouse conditions. The plants were grown in 20-cm diameter pots (five plants/pot) and they were maintained in greenhouse at ambient conditions (25 to 27°C, 14 h natural light). Pathogenicity tests were conducted on 17-day-old plants when they had at least three to four expanded leaves. The bacterial suspension consisted of 1×10^8 CFU/ml and was prepared from 48-h-old culture grown on NA medium. Bacterial suspensions were injected into the fully expanded leaves by a sterile insulin syringe (BD Lo-Dose, Fisher Scientific, Pittsburgh, PA), and the inoculated area was marked using a waterproof marker to ease the subsequent monitoring of the symptoms development on the leaves. Four plants were inoculated with each of the bacterial strains described in Table 1. The inoculated plants were maintained in greenhouse conditions with 90 to 95% relative humidity, and they were periodically monitored for symptom development.

Disease symptoms, i.e., water soaking, tissue discoloration, and lesion lengths, were measured at 48, 72, and 96 h postinoculation (hpi). Disease severity and strain aggressiveness were assessed using a lesion length index on an ordinal rating scale with five (0 to 4) symptom scales as follows: 0 = no disease symptoms, 1 = lesion length ≤ 2.0 cm, 2 = 2.1 to 5.0 cm, 3 = 5.1 to 8.0 cm, and 4 = lesion length > 8.0 cm. Disease severity was recorded in 24 h intervals starting from 48 hpi (i.e., 48, 72, and 96 hpi). Control plants were treated in the same manner, while standard strain of *X. translucens* pv. *undulosa* (ICMP 11055) and sterile distilled water were used as positive and negative controls, respectively. The strain ICMP 11055 was isolated from wheat in Southern Iran in 1983 and its TALome was recently investigated using the complete genomic DNA sequencing (Falahi-Charkhabi et al. 2017). Koch's postulates were accomplished by reisolating the inoculated strains on YDC medium from all the inoculated plants. Confirmation of the reisolated bacterial strains was made using *X. translucens*-specific PCR primers T1/T2 (CCGCCATAGGCGGAGCACCCCGAT/GCAGGTGCGACGTTTGCAGAGGGATCTTCTGCAA) as described by Maes et al. (1996). PCR procedure and parameters were the same as described previously (Khojasteh et al. 2019). The pathogenicity tests were repeated twice.

Disease severity index was calculated for each of the bacterial strains using the average lesion length on four inoculated plants. Data analysis was performed using a nonparametric procedure and conducted using the statistical package SPSS version 21 (IBM Corp., Armonk, NY). A dendrogram was constructed for disease severity of the *X. translucens* strains based on the ordinal score obtained from the monitoring of symptoms development at 48, 72, and 96 hpi. Strain clustering, based on disease severity, was performed using a simple matching coefficient similarity matrix based on a matrix of virulence level generated by the SIMQUAL routine in NTSYS (Rohlf 1998; Exeter Software, Setauket, NY). Cluster analysis was carried out using unweighted pair group mean algorithm (UPGMA) with the arithmetic mean method in the SAHN routine of NTSYS (Dice 1945; Jaccard 1908).

DNA extraction and Southern blotting. Total genomic DNAs of all 65 strains were extracted using the HiPure Bacterial DNA extraction Kit (Magen, Guangzhou, China) via the procedure recommended by the manufacturer. The quality and quantity of DNAs were spectrophotometrically evaluated and adjusted to 1.0 to 2.0 $\mu\text{g } \mu\text{l}^{-1}$ using Nanodrop ND-100 (Nanodrop Technologies, Waltham, MA) for further uses. DNA samples (50 μl /strain) were aseptically digested using *Bam*HI restriction endonuclease (Takara Bio, Kusatsu, Japan) at 37°C for 5 h. Denaturation and neutralization procedures were carried out as recommended by the manufacturer. Resulting DNA fragments were electrophoretically separated on 1.2% agarose gel in TAE (Tris-acetate-EDTA) buffer at 80V for 21 h in a cold chamber (4 to 6°C). Subsequently, to perform Southern blotting, they were transferred from the agarose gel onto positively charged Hybond N+ nylon membrane (Millipore, Billerica, MA) as recommended

TABLE 1. Information of *Xanthomonas translucens* strains isolated from different small grain cereals in Iran used in this study^a

Strain	Pathovar designation	Host of isolation	Province	Date	Genotype	Pathogenicity on	
						Wheat	barley
XtLr1	pv. <i>undulosa</i>	Wheat	Lorestan	2017	1	+	+
XtLr2	pv. <i>undulosa</i>	Wheat	Lorestan	2017	1	+	+
XtLr3	pv. <i>undulosa</i>	Wheat	Lorestan	2017	1	+	+
XtLr4	pv. <i>undulosa</i>	Wheat	Lorestan	2016	2	+	+
XtLr5	pv. <i>undulosa</i>	Wheat	Lorestan	2016	2	+	+
XtLr6	pv. <i>undulosa</i>	Wheat	Lorestan	2016	2	+	+
XtLr7	pv. <i>undulosa</i>	Barley	Lorestan	2017	2	+	+
XtLr8	pv. <i>undulosa</i>	Wheat	Lorestan	2016	1	+	+
XtLr9	pv. <i>undulosa</i>	Barley	Lorestan	2017	2	+	+
XtKr1	pv. <i>undulosa</i>	Wheat	Kermanshah	2016	2	+	+
XtKr2	pv. <i>undulosa</i>	Wheat	Kermanshah	2016	2	+	+
XtKr3	pv. <i>undulosa</i>	Wheat	Kermanshah	2016	2	+	+
XtKr4	pv. <i>undulosa</i>	Wheat	Kermanshah	2016	2	+	+
XtKr5	pv. <i>undulosa</i>	Wheat	Kermanshah	2016	2	+	+
XtZa1	pv. <i>undulosa</i>	Wheat	Zanjan	2016	4	+	+
XtZa2	pv. <i>undulosa</i>	Wheat	Zanjan	2016	4	+	+
XtZa3	pv. <i>undulosa</i>	Wheat	Zanjan	2016	4	+	+
XtZa4	pv. <i>undulosa</i>	Wheat	Zanjan	2016	4	+	+
XtZa5	pv. <i>undulosa</i>	Wheat	Zanjan	2016	4	+	+
XtFa1	pv. <i>undulosa</i>	Wheat	Fars	2016	6	+	+
XtFa2	pv. <i>undulosa</i>	Wheat	Fars	2016	6	+	+
XtFa3	pv. <i>undulosa</i>	Wheat	Fars	2016	6	+	+
XtFa4	pv. <i>undulosa</i>	Wheat	Fars	2016	6	+	+
XtFa5	pv. <i>undulosa</i>	Wheat	Fars	2016	6	+	+
XtFa6	pv. <i>undulosa</i>	Wheat	Fars	2016	7	+	+
XtFa7	pv. <i>undulosa</i>	Wheat	Fars	2016	7	+	+
XtKn1	pv. <i>undulosa</i>	Wheat	Kurdistan	2016	3	+	+
XtKn2	pv. <i>undulosa</i>	Wheat	Kurdistan	2016	3	+	+
XtKn3	pv. <i>undulosa</i>	Wheat	Kurdistan	2016	3	+	+
XtKn4	pv. <i>undulosa</i>	Wheat	Kurdistan	2016	3	+	+
XtKn5	pv. <i>undulosa</i>	Wheat	Kurdistan	2016	3	+	+
XtHn1	pv. <i>undulosa</i>	Wheat	Hamadan	2017	2	+	+
XtHn2	pv. <i>undulosa</i>	Wheat	Hamadan	2017	2	+	+
XtHn3	pv. <i>undulosa</i>	Wheat	Hamadan	2017	2	+	+
XtHn4	pv. <i>undulosa</i>	Wheat	Hamadan	2016	2	+	+
XtHn5	pv. <i>undulosa</i>	Wheat	Hamadan	2016	2	+	+
XtHn6	pv. <i>undulosa</i>	Wheat	Hamadan	2016	7	+	+
XtHn7	pv. <i>undulosa</i>	Wheat	Hamadan	2016	7	+	+
XtHn8	pv. <i>undulosa</i>	Wheat	Hamadan	2016	7	+	+
XtHn9	pv. <i>undulosa</i>	Barley	Hamadan	2017	7	+	+
XtHn10	pv. <i>undulosa</i>	Barley	Hamadan	2017	7	+	+
XtHn11	pv. <i>undulosa</i>	Barley	Hamadan	2017	7	+	+
XtKm3	pv. <i>undulosa</i>	Rye	Kerman	2015	4	+	+
XtKm4	pv. <i>translucens</i>	Barley	Kerman	2015	12	–	+
XtKm7	pv. <i>translucens</i>	Barley	Kerman	2014	Not assigned	–	+
XtKm8	pv. <i>translucens</i>	Barley	Kerman	2014		–	+
XtKm9	pv. <i>translucens</i>	Barley	Kerman	2015		–	+
XtKm10	pv. <i>undulosa</i>	Barley	Kerman	2015	4	+	+
XtKm11	pv. <i>translucens</i>	Barley	Kerman	2015	12	–	+
XtKm12	pv. <i>undulosa</i>	Wheat	Kerman	2015	8	+	+
XtKm15	pv. <i>undulosa</i>	Ryegrass	Kerman	2015	5	+	+
XtKm18	pv. <i>translucens</i>	Barley	Kerman	2015	9	+	+
XtKm22	pv. <i>undulosa</i>	Wheat	Kerman	2008	2	+	+
XtKm24	pv. <i>undulosa</i>	Wheat	Kerman	2008	2	+	+
XtKm25	pv. <i>undulosa</i>	Wheat	Kerman	2015	5	+	+
XtKm27	pv. <i>undulosa</i>	Wheat	Kerman	2015	2	+	+
XtKm28	pv. <i>undulosa</i>	Wheat	Kerman	2009	4	+	+
XtKm29	pv. <i>undulosa</i>	Wheat	Kerman	2015	4	+	+
XtKm31	pv. <i>undulosa</i>	Wheat	Kerman	2015	4	+	+
XtKm33	pv. <i>translucens</i>	Barley	Kerman	2009	10	–	+
XtKm34	pv. <i>translucens</i>	Barley	Kerman	2015	11	–	+
XtKm35	pv. <i>undulosa</i>	Wheat	Kerman	2015	4	+	+
XtBu36	pv. <i>undulosa</i>	Wheat	Kerman	2017	4	+	+
XtBu38	pv. <i>undulosa</i>	Wheat	Kerman	2017	4	+	+
ICMP 11055	pv. <i>undulosa</i>	Wheat	Kerman	ND	4	+	+

^a The pathovar designation, hosts, provinces, and years of isolation, as well as the pathogenicity on barley and wheat plants in greenhouse conditions. + indicates pathogenicity and – indicates nonpathogenicity.

by the manufacturer. The hybridization probe used in this study was a digoxigenin-labeled 1,707-bp DNA fragment originated from the internal *SphI* position of *talI* in *X. translucens* pv. *cerealis* strain NXtc01 (GenBank accession number PRJNA528834). This 1,707-bp probe contained 12 tandem repeats of a 102-bp DNA sequence of *talI* and part of the leucine zipper (LZ) coding region as described previously (Shah et al. 2019). The probe was used to detect the number and size of putative TALE genes in each *X. translucens* strain evaluated in this study. DNA labeling and hybridization was performed using DIG Easy hybridization buffer (Roche, Sweden) in a hybridization machine (Scientz, China) at 68°C for 2 h as recommended by the manufacturers. To detect and visualize the hybridized DNA fragments, 150 ml of detection buffer containing 0.1 M Tris, 0.1 M NaCl, and 50 mM MgCl₂ (Roche) was added to the membrane and incubated for 2 h in room temperature, while the resulted patterns were pictured using a portable digital camera. The Southern blotting assays were repeated twice.

The resulting digitized images were converted into TIFF files for the subsequent analysis of the blotting patterns. Analysis of putative TALEs was performed by making a binary form where 1 indicates the presence of a given band and 0 indicates the absence of the corresponding band. The intense hybridizations were considered double or triple bands, based on the intensity and thickness of the band, as described previously (Ji et al. 2014; Ye et al. 2013). Genetic similarity coefficients were calculated using similarity for qualitative data (SIMQUAL) with the Dice coefficient index (Dice 1945; Jaccard 1908). Results were subjected to cluster analysis using UPGMA within the sequential agglomerative hierarchical nested (SAHN) analysis module of NTSYS 2.02e (Rohlf 1998). Furthermore, the value of cophenetic correlation was assessed as measure of goodness of fit among matrices generated using the matrix comparison (MXCOMP)

module. The procedure was repeated twice independently to test the reproducibility of the blotting.

RESULTS

Virulence and aggressiveness of the strains. In order to investigate the virulence and aggressiveness of the *X. translucens* strains, they were inoculated onto barley and wheat plants, and lesion length was estimated at 48, 72, and 96 hpi. Considerable variations were observed among the aggressiveness of the strains (Fig. 1A to G). As for the *X. translucens* pv. *undulosa* strains, both the inoculated barley and wheat leaves showed water soaking symptoms at 24 hpi, while extended lesions were observed at 48 to 72 hpi. Water-soaking areas were turned into dark green to pale brown streaks at 72 to 96 hpi, indicating the aggressiveness of the corresponding strains (Fig. 1D to F). Bacterial exudates (ooze) were observed on the surface of symptomatic leaf tissues, forming yellow droplets on the leaf surface at 72 hpi, and turned into loose granules when they were exposed to the sunlight (Fig. 1G). On the other hand, virulence of the *X. translucens* pv. *translucens* strains was restricted to barley plants causing water soaking symptoms on the leaves at 24 to 48 hpi. On the wheat leaves inoculated with *X. translucens* pv. *translucens* strains, only faint discolorations (pale chlorotic areas as shown in Figure 1A) were observed on the sites of inoculation, which were not considered to be symptoms of BLS.

Disease severity analysis based on the 0 to 4 ordinal scoring of the virulence index on both barley and wheat plants has divided the 65 *X. translucens* strains into two main clades as shown in Figure 2. Clade one, which encompassed a bigger portion of the strains, consisted of both *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens* strains, while clade two consisted of only *X. translucens* pv. *undulosa* strains. All of the strains in clade two showed severe

Fig. 1. Symptoms of bacterial leaf streak disease caused by *Xanthomonas translucens* strains on artificially inoculated barley and wheat plants. Disease severity and strain aggressiveness were assessed using a lesion length index on an ordinal rating scale with five (0 to 4) symptom scales as follows: 0 = no disease symptoms (A, strain Xtkm7 on wheat), 1 = lesion length ≤2.0 cm (B, strain XtZa5 on barley and C, Xtlr6 on wheat), 2 = lesion length 2.1 to 5.0 cm (D, Xtkr4 on wheat), 3 = 5.1 to 8.0 cm (E, Xthn1 on wheat), and 4 = >8.0 cm (F, Xtkn5 on wheat). Under favored environmental conditions (e.g., high relative humidity), bacterial exudates (ooze) were observed on the surface of symptomatic leaf tissues forming yellow droplets on the leaf surface (G, XtfA6 on wheat).

aggressiveness on both barley and wheat plants where the virulence index of all the strains on both the plants was III or IV (Fig. 2). On the other hand, clade one was divided into two subclades (i.e., I-I and I-II) with considerable differences in disease severity indices between the two subclades (Fig. 2). Subclade I-I consisted of only *X. translucens* pv. *undulosa* strains, in all of which disease severity index was $\geq$ II at least in one of the plant species. However, subclade I-II included both the *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens* strains with an attenuated disease severity where the indices on barley and wheat plants were I and I, and I and 0, respectively.

The most severe aggressiveness was observed in strain XtKn5 with a mean lesion length of 9.0 and 8.5 cm on barley and wheat plants, respectively, followed by XtHn3 with a mean lesion length of 8.8 and 8.0 cm on barley and wheat plants, respectively

(Supplementary Table S1). On the other hand, XtKm12 and XtKm22 were the least virulent strains with a mean lesion length of 0.2 and 0.3 cm on wheat and barley plants, respectively. A number of *X. translucens* pv. *undulosa* strains showed variable virulence on barley and wheat plants. For instance, XtZa4, XtKm3, XtKm28, and XtKm29 were highly aggressive on wheat plants (score I) while they were slightly pathogenic on barley plants (score I) as shown in Figure 2. Symptoms of strains XtLr3 and XtLr8 on wheat plants were more severe (score III) than that observed on barley plants (score II). The inoculated bacterial strains were consistently reisolated from the symptomatic plants and their identity was confirmed using colony morphology on YDC medium, as well as the specific primer pair T1/T2 as described above. Negative control plants inoculated with sterile distilled water remained healthy, while the positive control plants inoculated with the reference strain

Fig. 2. Analysis of disease severity indices on barley and wheat plants inoculated with 65 *Xanthomonas translucens* strains in this study. Dendrogram on the left side shows clustering of the strains based on the ordinal scales of disease severity generated using the NTSYS software. Disease severity scale of each of the strains on both barley and wheat plants are indicated. Genotype designation is also presented for each of the strains based on transcription activator-like effectors diversity (Table 2). Furthermore, accumulated disease severity scales of the strains on barley and wheat plants at 48, 72, and 96 h postinoculation (hpi) are illustrated using the stacked bar graph. Shaded background indicates *X. translucens* pv. *translucens* (Xtt) strains.

ICMP 11055 showed water soaking symptoms on both the plant species at 24 hpi. Similar results were consistently observed in both replications of the pathogenicity tests.

TALE diversity. TALE diversity in the 65 *X. translucens* strains was estimated via Southern blotting using a hybridization probe based on the internal *SphI* fragment of *tal1* gene in *X. translucens* pv. *cerealis* strain NXtc01. Results of Southern blotting showed that all strains except for XtKm7 contained multiple fragments of putative TALE homologous to the *SphI* fragment of *tal1* (Fig. 3). The range of detected putative TALEs varied between 2.2 and 6.7 kb, while the number of detected fragments per strain varied from 5 to 12 (Fig. 3). Considering the number and size of the detected fragments, 65 *X. translucens* strains

were assigned into 13 genotypes as shown in Figure 4. It is also notable that the hybridized-TALE genes marked as A, B, C, D, E, and F seems the largest one in size present in the assigned genotypes except for XtKm7 (Fig. 4). Furthermore, diversity analysis using the NTSYS software confirmed the genotype assignment and clustered the strains into 12 clades (excluding strain XtKm7 in which no TALE was detected; Fig. 5). Interestingly, TALE diversity and clustering pattern was in congruence with the pathovar assignment and host range of the strains as described above. The 65 *X. translucens* strains were divided into two clades in 44% matching coefficient (Fig. 5). All 57 *X. translucens* pv. *undulosa* strains were placed in the same clade (clade I in Fig. 5) and divided into eight genotypes, while eight *X. translucens* pv. *translucens*

Fig. 3. Southern blot-based detection of putative transcription activator-like effectors (TALEs) in the 65 *Xanthomonas translucens* strains isolated in Iran. Genomic DNAs of the strains were digested using *Bam*HI restriction endonuclease and hybridized with an internal *SphI* fragment of *tal1* gene in *X. translucens* pv. *cerealis*. Each band represents a unique putative TALE, while the left lane (marker) shows the size of the detected fragments.

strains were placed in clade II and divided into five genotypes using NTSYS software (Fig. 5). As for the frequency of the strains among different genotypes, genotype 2 was the most populous group and contained 18 strains followed by genotype 4 which contained 14 strains. On the other hand, each of the genotypes 8, 10, 11, and 13 contained only one strain, followed by genotype 9, which contained two strains (Table 2).

TALE profiles in 65 *X. translucens* strains revealed pathovar-specific patterns within the strains evaluated in this study. For instance, the putative TALE fragment designated as M was present in all *X. translucens* pv. *undulosa* strains while absent in all *X. translucens* pv. *translucens* strains (Figs. 4 and 5). Furthermore, putative TALEs designated as A, B, D, H, I, S, T, U, and W were present in at least one *X. translucens* pv. *undulosa* strain but absent in all *X. translucens* pv. *translucens* strains. On the other hand, the putative TALEs designated as C, F, G, J, K, N, Q, and X were present in at least one *X. translucens* pv. *translucens* strain while absent in all *X. translucens* pv. *undulosa* strains. Interestingly, putative TALE fragment designated as R was common among all of the tested *X. translucens* strains, and the fragments E, L, O, P, and V were shared among at least one strain from each of the *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens* pathovars (Figs. 4 and 5; Table 2).

Several lines of evidences were detected indicating the congruence between the putative TALE profiles and disease severity index of the strains on barley and wheat plants. Six of the 13 TALE genotypes showed clustering patterns consistent with disease severity. For instance, the genotypes 9, 10, 11, and 12, which were assigned into the same *X. translucens* pv. *translucens* pathovar and the same clade in the TALE-based clustering, were also assigned into the same clade in the disease severity dendrogram

(Fig. 2). Furthermore, all the strains designated as genotypes 1 and 7 were clustered into subclade I-I of the disease severity dendrogram, while the genotypes 5, 6, and 8 were clustered in subclade I-II. However, the members of genotypes 2 and 4 were scattered through different clades of the dendrogram. As for the relationships between the TALE profile and geographic origin of the strains, all the strains isolated in Bushehr (two strains), Kermanshah (five strains), Kurdistan (five strains), and Zanjan (five strains) provinces were assigned into a unique genotype, while the strains isolated in Fars (seven strains), Hamadan (11 strains), and Lorestan (nine strains) provinces were assigned into two different genotypes (Table 3). Interestingly, the strains isolated in Kerman province (21 strains) were scattered through nine different genotypes, indicating higher genetic diversity of the pathogen in this province (Table 3).

DISCUSSION

In this study, we provide a TALE-based genetic diversity scheme for 65 *X. translucens* strains isolated in eight cereal-growing provinces in Iran. Results obtained in this study helped us to bridge the knowledge gaps of whether TALE diversity is most closely associated with phylogeny, virulence, host range, or geographic region, and whether either TALE diversity or presence of specific TALEs might be predictive of virulence in *X. translucens*. All 65 strains were assigned into 13 genotypes based on the size and number of putative TALE fragments detected using Southern blotting. Further, based on the TALE diversity, the strains were divided into two main clusters, where cluster one encompassed 57 strains (genotype 1 to 8) and cluster two included seven strains (genotype 9 to 12). TALE-based clustering was in congruence with the MLSA-based phylogeny of the same strains using the sequences of four housekeeping genes (i.e., *dnaK*, *fyuA*, *gyrB*, and *rpoD*) as reported previously (Khojasteh et al. 2019). Accordingly, 57 *X. translucens* pv. *undulosa* strains were assigned into cluster one and seven *X. translucens* pv. *translucens* strains were placed in cluster two. Surprisingly, we did not find any TALEs in the strain XtKm7, which was previously identified as *X. translucens* pv. *translucens* using MLSA. The strain XtKm7 was designated as genotype 13 apart from all the TALE-harboring strains. On the other hand, we found that some, but not all of the strains clustered in TALE effector groups corresponding to virulence groups, while other TALE effector groups had no relation to virulence.

Recently, complete genome sequencing accomplished with the mutagenesis and complementation analyses revealed that the Iranian *X. translucens* pv. *undulosa* strain ICMP 11055 harbors seven TALE genes (Falahi-Charkhabi et al. 2017), as is the case of genotype 4 consisting of 14 *X. translucens* pv. *undulosa* strains in this report (Figs. 3, 4, and 5; Table 2). Results of Southern blot hybridization using the same strain indicate the presence of seven TALEs in the strain ICMP 11055, confirming the accuracy and validity of the procedure used in this study. According to the results of Southern blotting, we indicate that the BLS pathogen in Iran possesses high genetic diversity in TALEs profile and aggressiveness of the strains on barley and wheat plants. For instance, the whole genome sequencing of an Iranian *X. translucens* pv. *translucens* UPB886 (Roman-Reyna et al. 2019) and the pathotype strain of the pathogen DSM 18974^T isolated in the United States (Jaenicke et al. 2016) showed five and eight TALEs, respectively. However, the *X. translucens* pv. *translucens* strains evaluated in this study were assigned into five genotypes, indicating higher genetic diversity of the pathovar than that has so far been elucidated.

Higher genetic diversity and higher sequence type/multilocus haplotype frequency were also observed in the MLSA-based evaluation of the same population when compared with all the available worldwide *X. translucens* strains (Khojasteh et al. 2019). Furthermore, the highest TALE diversity was observed among the strains isolated in Kerman province (Table 3). Kerman province is located in Southern Iran close to the Fertile Crescent territory.

Fig. 4. Southern blot patterns in the representative *Xanthomonas translucens* strains from 13 genotypes based on transcription activator-like effectors (TALEs) diversity of the strains. All the representative strains except for XtKm7 contained multiple fragments of putative TALEs. Among the 13 genotypes, eight genotypes were represented by *X. translucens* pv. *undulosa* strains (i.e., strains XtFa1, XtHn3, XtHn8, XtKm12, XtKm15, XtKn5, XtLr8, and XtZa4), while five genotypes (i.e., strains XtKm33, XtKm34, XtKm7, XtKm8, and XtKm9) belonged to *X. translucens* pv. *translucens*. Each putative unique TALE fragment in the Southern blotting profile was indicated with a unique letter (A to X on the right side of image).

Fertile Crescent is known as “cradle of civilization” where the settled farming first began to emerge and people started to grow newly domesticated small grain cereals (Beck et al. 1999). These observations were in congruence with the hypothesis that plant pathogens have higher genetic diversity in the center of origin of their host plants.

In conclusion, this study provides a novel insight into the virulence-associated genetic diversity of the BLS pathogen in Iran, and elucidates relationships between the aggressiveness and TALE repertoires of the *X. translucens* strains isolated in the country. Unlike the previous population diversity investigations, which were mostly performed using either non-virulence-associated regions of

the bacterial genomic DNA (e.g., REP, ERIC, and BOX) (Adhikari et al. 2012; Bragard et al. 1995; Kölliker et al. 2006; Rademaker et al. 2005, 2006) or the sequences of housekeeping genes (Khojasteh et al. 2019), this study relied on the virulence-associated features (TALEs) of the *X. translucens* strains, which may be contributed to the epidemiology and upcoming outbreaks of the disease. Our results highlight the higher genetic diversity of BLS pathogen than that has so far been described in Iran, and underlines at the same time the potential consequences of high variability in the virulence-associated genetic contents of the pathogen in the country. On the other hand, our results emphasize the need to develop new and state of the art detection techniques to prevent the international/intercontinental

Fig. 5. Southern blot-based transcription activator-like effector (TALE) diversity in 65 *Xanthomonas translucens* strains generated using the unweighted pair group mean algorithm (UPGMA) implemented in the NTSYS software. All 65 strains were clustered into two main clades, which were in congruence with the pathovar designation of the strains based on their host range. Fifty-seven *X. translucens* pv. *undulosa* strains (Table 1) were placed in clade one (including eight genotypes designated as 1 to 8), while eight *X. translucens* pv. *translucens* strains were clustered in clade two including four genotypes (9 to 12). Disease severity index on barley and wheat plants, as well as multilocus haplotype designation of the strains obtained in the previous study (Khojasteh et al. 2019), were presented for each of the strains. Black and white section on the right side of the image shows the TALE profile of the strains as shown in Figure 3. Each column represents a unique putative TALE fragment designated from A to X (as described in Fig. 4), where black squares indicate the presence of a given fragment and white squares indicate the absence of the fragment. Shaded background indicates *X. translucens* pv. *translucens* strains.

TABLE 2. Classification of 65 *Xanthomonas translucens* strains isolated in Iran based on the transcriptional activator-like effector (TALE) diversity obtained from southern blotting

Genotype	Detected fragments ^a	Strains	Number of strains	Number of detected TALE genes
1	DMRSTVW	XtLr1, XtLr2, XtLr3, XtLr8	4	7
2	DMPRSTV	XtLr4, XtLr5, XtLr6, XtLr7, XtLr9, XtKr1, XtKr2, XtKr3, XtKr4, XtKr5, XtHn1, XtHn2, XtHn3, XtHn4, XtHn5, XtKm22, XtKm24, XtKm27	18	7
3	AMPRSTV	XtKn1, XtKn2, XtKn3, XtKn4, XtKn5	5	7
4	DMPRSTU	XtZa1, XtZa2, XtZa3, XtZa4, XtZa5, XtKm3, XtKm10, XtKm28, XtKm29, XtKm31, XtKm35, XtBu36, XtBu38, ICMP11055	14	7
5	EMPRSTU	XtKm15, XtKm25	2	7
6	DILMPRV	XtFa1, XtFa2, XtFa3, XtFa4, XtFa5	5	7
7	BHMPRSU	XtFa6, XtFa7, XtHn6, XtHn7, XtHn8, XtHn9, XtHn10, XtHn11	8	7
8	EHMOPRS	XtKm12	1	7
9	CGJKLRVX	XtKm8, XtKm18	2	8
10	EFGJKLOPQRVX	XtKm33	1	12
11	FJKLRVX	XtKm34	1	7
12	FNQRV	XtKm4, XtKm9, XtKm11	3	5
13	None	XtKm7	1	0

^a Designation of the strains into a given genotype was based on the absence/presence of a given fragment (A to X, as shown in Figure 4) in the Southern blot patterns as shown in Figure 3. The number of detected fragments (A to X) and the number of strains in each genotype are presented separately.

TABLE 3. Relationships between transcriptional activator-like effector diversity and geographic distribution of *X. translucens* strains isolated in Iran^a

Province	Genotypes													Number of strains
	1	2	3	4	5	6	7	8	9	10	11	12	13	
Lorestan	4	5	–	–	–	–	–	–	–	–	–	–	–	9
Kermanshah	–	5	–	–	–	–	–	–	–	–	–	–	–	5
Zanjan	–	–	–	5	–	–	–	–	–	–	–	–	–	5
Fars	–	–	–	–	–	5	2	–	–	–	–	–	–	7
Kurdistan	–	–	5	–	–	–	–	–	–	–	–	–	–	5
Hamadan	–	5	–	–	–	–	6	–	–	–	–	–	–	11
Kerman	–	3	–	7	2	–	–	1	2	1	1	3	1	21
Bushehr	–	–	–	2	–	–	–	–	–	–	–	–	–	2
Total	4	18	5	13	2	5	8	1	2	1	1	3	1	65

^a The number of corresponding strains in each genotype in a given province was indicated. – indicates the lack of a given genotype.

spread of the pathogen via infected cereal seeds from Iran, where is the center of origin of the host plants and center of diversity of the pathogen.

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