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TALome and phenotypic analysis of Pakistani *Xanthomonas oryzae* pv. *oryzae* population revealed novel virulent TALEs contributing to bacterial blight of rice

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Abstract

Bacterial blight (BB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is an important disease in rice-growing countries, including Pakistan, where it was first reported in the mid-1970s. Transcription activator-like effectors (TALEs) play vital roles in many plant diseases caused by *Xanthomonas* spp.; however, Pakistani *Xoo* TALome diversity and their contribution to pathogenicity is largely unknown. In this study, 101 *Xoo* strains were screened using specific PCR primers. The genomic DNA from these strains underwent *Bam*HI digestion and hybridized with the internal *Sph*I fragment of *PthXo1*. Southern blot analysis revealed 16 to 20 putative *tale* fragments among the tested strains. These strains were further classified into 11 genotypes based on the number and size of the hybridizing bands. Genotypes 1, 2, 3, and 4 represented 24, 2, 51, and 17 strains, respectively. Pathogenicity assays on near-isogenic lines (NILs) containing different resistance (*R*) genes exhibited that CBB23 was incompatible with all tested Pakistani-*Xoo* genotypes, whereas IRBB5 and IRBB4 showed resistance against specific genotypes. In contrast, paddy trails on NILs containing single, double, and triple mutants of *OsSWEET11a*, *OsSWEET13*, and *OsSWEET14* in the effector binding elements (EBEs) of cv. Kitaake revealed that KP-22 and LD-5 harbor novel virulent TAL effector/s. Interestingly, the expression analysis of six clade-III *OsSWEET* genes suggests that novel TALE/s targeting unidentified susceptibility gene/s. Altogether, this study highlights gene-for-gene relationships between tested rice lines and Pakistani-*Xoo* strains. This is the first report providing the diversity of TALEs and their relationship to *R* and *S* (susceptibility) genes. Further identification of novel virulent TALE/s and their cognate target/s is warranted to precisely elucidate their role in BB.

Keywords *Xanthomonas oryzae* pv. *oryzae*, Bacterial blight, TALome diversity, Major TALEs EBE edited susceptibility genes, NILs containing *R* genes

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Background

Rice (*Oryza sativa* L.) is one of the vital staple foods for over half of the world's population, which meets both calories and food requirements (Ainsworth 2008). Addressing the intricate environmental challenges and escalating global population, there is a pressing need for concerted efforts to stabilize rice production, although rice production has increased over the past decade (FAO). However, this agricultural intensification brings with it the potential threat of emerging pathogens, posing a significant risk unless locally adapted control solutions are promptly implemented (Gregory et al. 2009).

Among foliar diseases, bacterial blight (BB), caused by the pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is an important disease of rice worldwide. It limits rice production each year owing to its high epidemic potential in tropical regions, especially in Southern Asia and parts of West Africa (Nino-Liu et al. 2006). *Xoo* enters the leaf through hydathodes or wounds, causing systematic infection of the vascular system. This results in green, small water-soaked spots at the margins and tips of leaves, which expand along the veins, merge, and become chlorotic and then necrotic lesions (Ou 1985; Nino-Liu et al. 2006). Reported yield losses due to BB range from 20 to 30%, with reductions as high as 50–90% in some areas (Ou 1973; Nino-Liu et al. 2006; Liu et al. 2014). Moreover, rice BB is reported as an emerging disease worldwide, seriously affecting the quality and quantity in almost all of the rice growing countries (Naqvi 2019).

Similar to other gram-negative bacteria, *Xoo* employs a type-III secretion system (T3SS) to translocate a cocktail of type-III effectors (T3Es) into the host cell cytoplasm. These T3Es can be categorized into transcription activator-like effectors (TALEs) and non-TALEs or *Xanthomonas* outer proteins (Xops). Unlike the diverse molecular activities found in Xops, members of the TALEs family resemble eukaryotic transcription factors and exhibit sequence-specific binding to the promoters of target genes within the host cells. The specific sequences targeted by TALEs are referred to as the effector binding elements (EBEs). Structurally, TALEs consist of an N-terminal domain containing a type III secretion signal followed by a central repeat region (CRR; 33–35 amino acids [aa], in which 12th and 13th aa are variable and called repeat variable di-residue; RVD), and a C-terminus domain harboring nuclear localization signal (NLS) and transcriptional activation domain (AD) that are important for effector localization and gene activation, respectively (Boch and Bonas 2010; Mak et al. 2013; Richter et al. 2014; Perez-Quintero and Szurek 2019).

In general, following injection, TALE proteins localize into the host cell nucleus, where they recognize and bind to their EBEs in the promoter region of host resistance (*R*) or susceptibility (*S*) genes, inducing their expression (Bogdanove et al. 2010). Based on a handful of characterized examples, TALEs exploit host *S* genes, which play important roles in host recognition, subvert plant immunity, and transport nutrients to the pathogen. Most of the known TALE-targeted *S* genes encode transporters and transcriptional factors. For example, three specific sugar transporter genes, belonging to clade-III of *SWEET* genes, are targeted by about ten major known TALEs, including *OsSWEET11a*/*Xa13*/*Os8N3* induced by PthXo1 and Tal6b/AvrXa27A at overlapping EBEs (Yang et al. 2006; Xu et al. 2023), *OsSWEET13*/*Xa25*/*Os12N3* targeted by multiple PthXo2-like TALEs (i.e., PthXo2, PthXo2C/Tal5$_{LN18}$, PthXo2B/Tal7$_{PXO61}$, and Tal7$_{K74}$) (Zhou et al. 2015; Xu et al. 2019), and *OsSWEET14*/*Xa41*(*t*)/*Os11N3* activated by PthXo3, Tal5/TalF, TalC, and AvrXa7 at overlapping/different EBEs (Antony et al. 2010; Yu et al. 2011; Streubel et al. 2013; Tran et al. 2018). Recently, a new rice *S* gene (*OsERF#123*) was shown to be targeted by TalB in the African strains of *Xoo* (Tran et al. 2018). In addition, *X. oryzae* pv. *oryzicola* Tal2g targets *OsSULTR3;6*, a sulfate transporter gene, which is a major *S* gene for bacterial leaf streak (Cernadas et al. 2014). Another class of TALE-dependent *S* genes comprises host transcription factors; for instance, PthXo6 and PthXo7 of *Xoo* induce the expression of rice transcription factors *OsTFX1* and *TFIIAγ1*, respectively (Sugio et al. 2007).

In response to the action of TALEs, plants have evolved effective strategies to trigger resistance, such as loss of *S* genes or use of *R* genes (Kourelis and Van Der Hoorn 2018). Amongst 47 *R* genes, 17 have been cloned that individually confer rice resistance against BB (Jiang et al. 2020). Eight of these genes are TALE-dependent, including four recessive and four dominant *R* genes (*xa5*, *xa13*, *xa25*, *xa41*, *Xa7*, *Xa10*, *Xa23*, and *Xa27*), reflecting the crucial role of TALEs in the interaction (Hutin et al. 2015; Ji et al. 2022). The recessive *R* genes involve mutations within the EBEs of *S* genes to prevent TALE-DNA interactions. For example, *xa13* (*OsSWEET11a*), *xa41* (*t*) (*OsSWEET14*), *xa25* (*OsSWEET13*), and *xa5* (TFIIAγ5) cannot induce susceptibility due to mutations in the promoter EBEs or amino acid sequences (Chu et al. 2006; Gu et al. 2009; Liu et al. 2011). TALEs mediate executor (*E*) genes of so-called dominant resistance, that restrict the growth of the pathogen via rapid cell death. For instance, rice executor *R*-genes *Xa7, Xa10, Xa23,* and *Xa27* are induced by their matching TALEs, AvrXa7, AvrXa10, AvrXa23, and AvrXa27, respectively (Gu et al. 2005; Tian et al. 2014; Wang et al. 2015; Chen et al. 2021). These TALEs are exclusive to Asian *Xoo* strains, and none of the *E* genes have been induced by African strains. The *E* genes encode small proteins with transmembrane

domains, and the molecular mechanisms governing their function remain largely unclear (Zhang et al. 2015; Chen et al. 2021). Moreover, receptor-like kinase (RLK) genes, such as *Xa3*/*Xa26*, *Xa4*, and *Xa21*, also confer dominant resistance (Song et al. 1995; Sun et al. 2004; Hu et al. 2017).

Direct protein–protein (TALE and NBS-LRR proteins) interaction is responsible for another type of disease resistance, independent of direct host gene activation, for example, rice *Xa1* and *Xo1* (Yoshimura et al. 1998; Triplett et al. 2016; Xue et al. 2020). To escape host immunity, pathogenic bacteria have developed and deployed several different strategies such as truncTALEs (truncated TALEs) or iTALEs (interfering TALEs) to restrict TALE-dependent plant defense responses; for example, iTALE/trucTALE Tal2h suppresses *Xa1*/*Xo1* mediated resistance (Ji et al. 2016; Read et al. 2020; Xu et al. 2021). After decades of this effort, the mode of action of TALE proteins and plant resistance has taught us a valuable lesson about the never-ending co-evolutionary arms race during plant–microbe interactions.

In *Xoo*, there is an uneven distribution of TALE repertoires, ranging from 8 to 21 (African *Xoo* 8–9 while Asian *Xoo* 18–21), but very few of them are characterized (Tran et al. 2018; Oliva et al. 2019). Therefore, there is a need to explore the reasons behind the abundance of TALEs in *Xoo* and understand their role in promoting virulence. The TALE repertoires are structured into gene clusters along the chromosome, and it is proposed that the evolution of *tal* genes occurs through the mutation in the repeat sequences, rearrangement, and/or the deletion of single repeats (Erkes et al. 2017). Accordingly, certain repeat arrays have been identified among unrelated TALEs within the same strain, indicating the possibility of intergenic recombination as a mechanism for generating novel variants (Tran et al. 2018). Due to their involvement in pathogenicity and interaction with corresponding *R* genes in rice varieties, the evolution of TALEs is subjected to continuous selective pressure, leading to rapid evolutionary changes (Schandry et al. 2018). Knowing the TALomes diversity and understanding the evolutionary patterns is essential for strategically deploying varieties equipped with locally adapted resistance genes and anticipating the risk of the emergence of new, highly aggressive strains.

Resistance gene deployment requires knowledge of the bacterial population structure and their spatiotemporal variability. Therefore, the main purpose of this study was to evaluate the TALome diversity of *Xoo* strains collected from two provinces in Pakistan and investigate gene-for-gene interactions between *Xoo* and rice to improve their control. Here, we present the genetic and pathogenic characterization of 101 Pakistani *Xoo* strains collected in 2017, 2018, 2019, and 2021. TALE repertoires profiling revealed the presence of 16–20 putative TALE fragments ranging from about 2.2 kb to 4.6 kb. Pathotyping on NILs containing different *R* genes indicated that CBB23 is effective against all the tested strains. On the other hand, NILs containing EBE mutants of *OsSWEET11a*, *OsSWEET13*, and *OsSWEET14*, revealed that KP-22 and LD-5 harbor a novel virulent TAL effector/s. Furthermore, expression analysis of six clade-III *OsSWEET* genes suggested the presence of novel TALE/s targeting previously unidentified susceptibility gene/s. This report represents the first comprehensive analysis of TALE diversity and its association with *R* and *S* genes. Taken together, our approach highlighted that Pakistani-*Xoo* strains contain novel virulent TALEs targeting novel gene/s. Further identification of novel virulent TALEs is imperative for a precise understanding of their role in BB.

Results

Collection of Pakistani-*Xoo* strains

Rice growing areas of Punjab (P) and Khyber Pakhtunkhwa (KP) were surveyed in 2017, 2018, 2019, and 2021 (Additional file 1: Table S1). Five districts each in Punjab and Khyber Pakhtunkhwa were sampled where bacterial blight was present in varying intensity. In Punjab, the disease incidence was low in 2017 and 2018 compared to 2019 and 2021. The highest disease incidence (80%) was recorded in Sialkot during 2021, followed by Narowal (50%) and Gujranwala (36%). The disease was also observed in other Basmati growing areas such as Hafizabad and Sheikupura. In KP, the disease incidence was comparatively lower than in Punjab. The highest disease incidence (40%) was recorded in Swat in 2018, followed by Lower Dir, Battagram, Mansehra, and Bannu (Additional file 2: Figure S1).

To assess the diversity of TALE repertoires and pathogen aggressiveness, a collection of 101 *Xoo* strains were isolated, among which 39 were from Punjab and 62 from KP. In total, 40 strains were isolated from leaves collected in 2017, 22 in 2018, 24 in 2019, and 15 in 2021 (Additional file 2: Figure S1). The strains grow as pale yellow to brownish colonies on NA plates and cause disease on rice upon inoculation. Moreover, the pathovar-specific PCR primers Xoo80F/Xoo80R amplify a 162 bp DNA fragment, confirming that all the strains are *Xoo* (Additional file 1: Table S2). Additionally, the product size and intensity were also similar in gel compared with PXO99^{A} (Additional file 2: Figure S2).

TALes diversity in Pakistani-*Xoo* strains

To investigate the TALome diversity of 101 *Xoo* strains isolated from the rice growing fields in Pakistan (Additional file 2: Figure S1), we conducted Southern blot

(SB) hybridization. The well-characterized Philippines and Chinese strains, PXO99[A] and LN18, were included for comparative purposes. The SB results revealed that all the strains exhibited multiple fragments of putative TALEs homologous to the *Sph*I fragment of PthXo1 (Additional file 2: Figure S3). The detected putative TALEs ranged in size from about 2.2 to 4.6 kb, and the number of fragments per strain varied from 16 to 20, considering the intensely hybridizing bands as multiple fragments (Table 1). Based on the size and number of the detected TALE fragments, the 101 strains were classified into 11 genotypes. The hybridized *tale* genes were indicated as A to T in descending order (Fig. 1). The TALE profiles of 11 genotypes (hereafter refer as G1, G2, and so on) of Pakistani-*Xoo* strains revealed geographic distinct pattern except for G3 and G4. Notably, the putative TALE fragments designated as D, E, F, L, O, and Q were consistently present in all *Xoo* strains. The intensely hybridizing fragments were considered as two and four bands, based on the intensity, thickness, and comparison (*in-silico* simulation on gel) with the available Pakistani *Xoo* strain PkXoo1 (TALEs = 18), which revealed multiple fragments on the same position. TALE fragments D = D1, D2; E = E1, E2; L = L1, L2, L3, L4 (in all genotypes); O = O1, O2 (in G1 to G6, G8, G10, and G11); Q = Q1, Q2 (in G7 to G11), and R = R1, R2 (in G8, G10, and G11) are intensely hybridizing and were considered multiple fragments. TALEs designated as H, I, J, K, M, N, P, and R were detected in more than two genotypes. Furthermore, the TALE fragments designated as A, B, C, G, S, and T were uniquely present in genotypes G2, G7, G11, G5, G6, and G8, respectively (Fig. 1, Table 1).

TALE diversity analysis by clustering the presence and absence of *Bam*HI fragment using NTSYS 2.02e tool classified the 11 genotypes into 5 clades (Fig. 2). Interestingly, the clade pattern was almost in congruence with the geographic distribution of the strains, i.e., genotypes from Punjab G1, G2, and G4; G6 grouped into clade-1 and clade-3, respectively. The other genotypes belong to KP, i.e., G3 and G5; G8, G11, and G10 were grouped into clade-2 and clade-5, correspondingly (Fig. 2). Genotypes G7 and G9, each consisting of a single strain from

Table 1 Transcriptional activator-like effector (TALE) genotypes of *Xanthomonas oryzae* pv. *oryzae* strains collected from different rice-growing regions of Pakistan

Genotypes	Strains names	Total strains	Detected putative *tale* fragments	Total *tale* fragments
G1	P-10, P-11, P-31, P-23, P-24, P-26, P-28, P-29, P-20, P-30, P-38, P-8, P-9, P-10, P-11, P-12, P-25, P-5, P-6, P-7, P-22, P-17, P-25, P-28	24	D1, D2, E1, E2, F, H, I, L1, L2, L3, L4, M, N, O1, O2, Q	16
G2	P-14, P-19	2	**A**, D1, D2, E1, E2, F, H, I, L1, L2, L3, L4, M, N, O1, O2, Q	17
G3	KP-3, KP-8, KP-9, KP-11, KP-16, KP-23, KP-29, KP-31, BG-1, BG-2, BG-5, BG-22, BN-6, LD-67, LD-35, LD-51, BN-7, BN-10, BN-13, BN-17, BN-18, BN-25, BG-25, BG-27, BG-32, BG-39, LD-3, P-17, P-32, LD-24, LD-31, LD-33, LD-36, LD-41, LD-46, LD-61, LD-71, LD-55, BN-12, BG-24, LD-2, KP-1, KP-2, KP-17, KP-34, LD-21, BG-8, LD-17, LD-20, LD-22, LD-42	51	D1, D2, E1, E2, F, H, I, K, L1, L2, L3, L4, M, O1, O2, Q, R	17
G4	P-35, P-36, P-37, P-25(Old), P-4, P-13, KP-10, KP-12, KP-13, KP-14, KP-18, KP-20, KP-21, P-23(Old), KP-15, P-15, P-16	17	D1, D2, E1, E2, F, H, I, K, L1, L2, L3, L4, M, N, O1, O2, Q	17
G5	LD-13	1	D1, D2, E1, E2, F, **G**, H, K, L1, L2, L3, L4, M, O1, O2, Q, R	17
G6	P-27	1	D1, D2, E1, E2, F, H, J, K, L1, L2, L3, L4, N, O1, O2, Q, **S**	17
G7	P-18	1	**B**, D1, D2, E1, E2, F, H, I, L1, L2, L3, L4, M, N, O, P, Q1, Q2	18
G8	*KP-22*	*1*	*D1, D2, E1, E2, F, I, J, K, L1, L2, L3, L4, M, O1, O2, Q1, Q2, R1, R2,* ***T***	*20*
G9	KP-24	1	D1, D2, E1, E2, F, I, J, K, L1, L2, L3, L4, M, N, O, P, Q1, Q2	18
G10	BG-3	1	D1, D2, E1, E2, F, I, J, K, L1, L2, L3, L4, O1, O2, Q1, Q2, R1, R2	18
G11	*LD-5*	*1*	***C****, D1, D2, E1, E2, F, I, J, K, L1, L2, L3, L4, M, O1, O2, Q1, Q2, R1, R2*	*20*

Strain classification into a given genotype was determined based on the presence/absence of *tale* fragments (labeled A–T, as shown in Fig. 1). The number of strains, detected fragments, and the total number of *tale* fragments in each genotype are presented separately. Fragments displaying intense hybridization were considered two and four bands, considering the intensity, thickness, and comparison with the single available Pakistani *Xoo* strain PkXoo1 (TALEs = 18), which exhibited multiple fragments at the same position. Specifically, TALE fragments D = D1, D2; E = E1, E2; L = L1, L2, L3, L4 (common to all genotypes); O = O1, O2 (in G1–G6, G8, G10, G11); Q = Q1, Q2 (in G7–G11), and R = R1, R2 (in G8, G10, G11) demonstrated intense hybridization, and were considered multiple fragments. Unique TALE fragments are shown with bold fonts and the genotypes/strains containing novel major virulent TALE/s are highlighted italic font

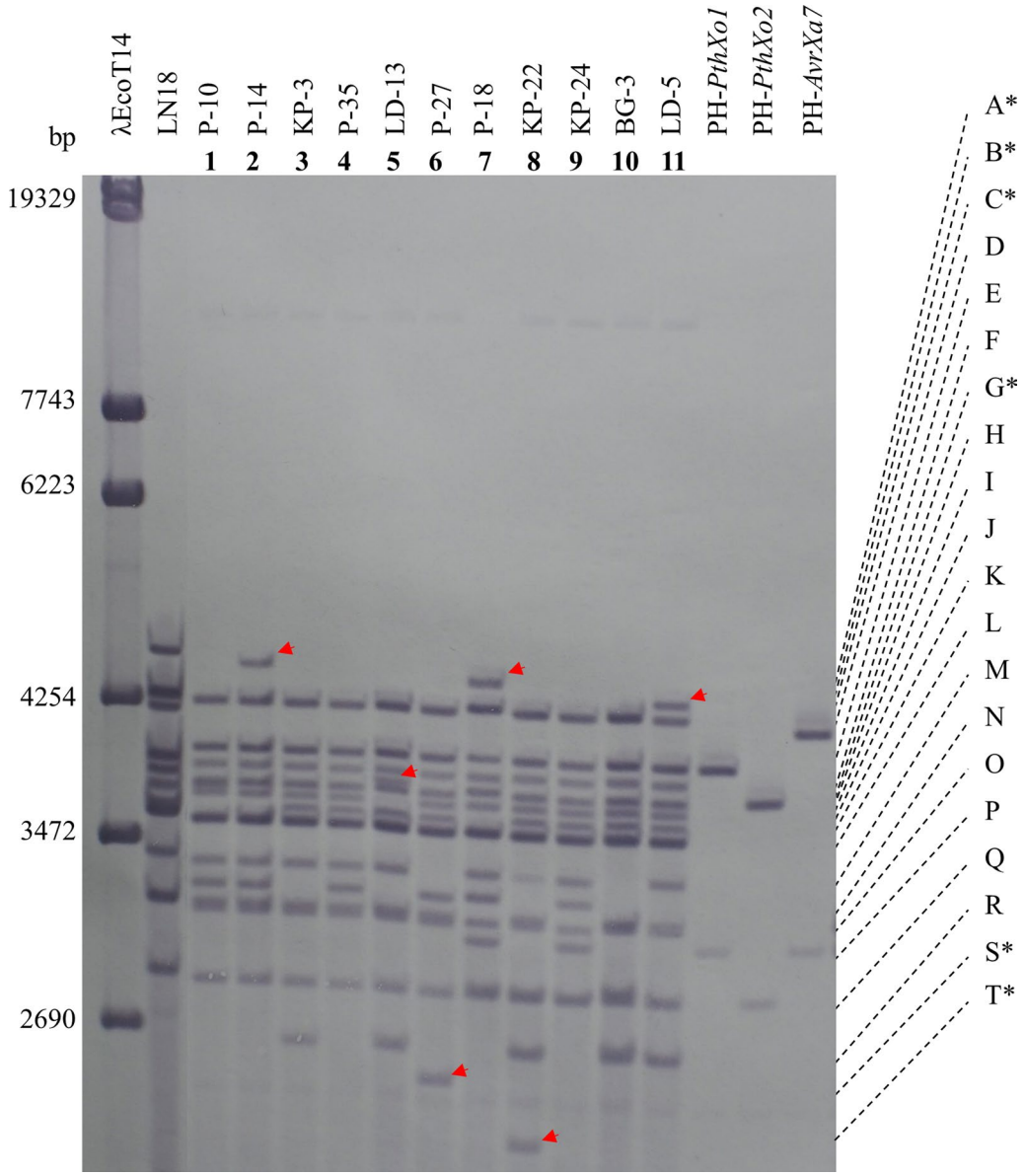

Fig. 1 Southern blot patterns depicting the 11 genotypes of Pakistani *Xoo* isolates. Genomic DNAs from the mentioned strains underwent *Bam*HI digestion and were subsequently hybridized with an internal *Sph*I fragment of *PthXo1*. The 101 strains were categorized into 11 distinct genotypes: G1 (24 strains), G2 (2 strains), G3 (51 strains), G4 (17 strains), and G5 to G11, each containing a single strain, respectively (Table 1). Genotypes are displayed at the top of the image beneath the strain names. The hybridizing bands of varying sizes are identified by different letters (A to T) on the right side of the figure, while bands marked with an asterisk (*) were unique to a single strain (pointed out with the red arrows in the figure). TALE fragments D = 2, E = 2, L = 4 (in all genotypes); O = 2 (G1 to G6, G8, G10, and G11), Q = 2 (G7 to G11), and R = 2 (G8, G10, and G11) are intensely hybridizing and were considered multiple (Table 1). Strain names were assigned using a letter representing their province of origin, followed by a number, except for LD (Lower Dir, KP), BG (Battagram, KP), and BN (Bannu, KP) strains. The province abbreviations used are as follows: P for Punjab and KP for Khyber Pakhtunkhwa. The left lanes of the image feature the λEcoT14 marker (base pairs; bp) and strain LN18 for reference. The right lanes represent the PH strain (PXO99^{A} *tal*-free) containing *PthXo1*, *PthXo2*, and *AvrXa7 in trans* that were used to highlight the major virulence *tal* genes

Punjab and KP, were grouped into clade-4. Regarding the distribution of strains across different genotypes, G3 is the most common group containing 51 strains (49 strains from KP and 2 strains from Punjab). Other genotypes, G1 and G4 (9 strains from Punjab and 8 strains from KP), also contained many strains, i.e., 24 and 17 strains, respectively. On the other hand, genotypes G5, G6, G7, G8, G9, G10, and G11 each contained an individual

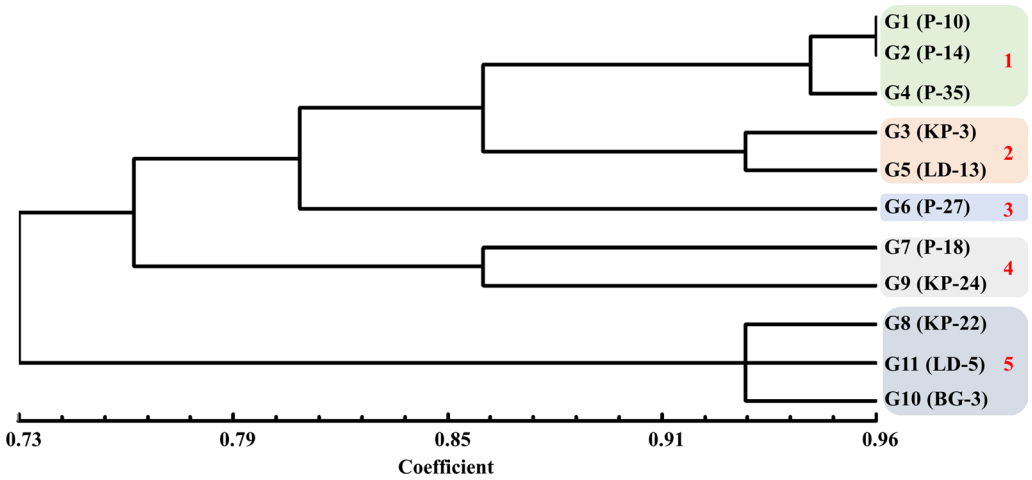

Fig. 2 Dendrogram illustrating the relatedness of Pakistani *Xoo* genotypes. The dendrogram demonstrates the relationship of *Xoo* genotypes based on Southern blot analysis, considering the presence or absence of hybridizing *Bam*HI fragments (Fig. 1). All genotypes were classified into 5 clades (1–5, in red fonts) as block shaded. The tree was generated by creating the similarity matrix in Simqual and clustering in SAHN using the NTSYSpc-2.02e. Genotypes are presented with their representative reference strain in parentheses

strain, followed by G2, which contained two strains. Moreover, the genotypes G3 and G4 have strains from both Punjab and KP as described above (Table 1). Additionally, our results indicated that strains collected from Sialkot and Swat are more diverse than those collected from other regions (Table 2).

To exploit the major virulence *tal* genes in SB, we used PH strain (PXO99^{A} *tal*-free) containing *PthXo1*, *PthXo2*, and *AvrXa7 in trans*. Southern blot analysis revealed that all the genotypes contained similar fragments of *PthXo1* and seven genotypes (G1, G2, G3, G4, G9, G10, and G11) harbor potential fragments of *PthXo2*, while the *AvrXa7* fragment did not match with any of the genotypes (Fig. 1). Interestingly, the majority of the strains contained both *PthXo1* and *PthXo2*, which implies that these strains possess the capability to simultaneously activate *OsSWEET11a* and *OsSWEET13*. Notably, SB results did not completely align with the inoculation phenotypes, possibly due to the presence of similar-sized TALEs that remained unidentified.

Germplasm screening for TALes-dependent resistance

To assess the prevalence and diversity of TALes counter resistance, 11 genotypes (a collection of 101 strains)

Table 2 Genotypes of *Xanthomonas oryzae* pv. *oryzae* strains collected from different localities of Pakistan

Province	Districts	Genotypes*											
		G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	Strains#
Khyber Pakhtunkhwa (62)	Bannu	–	–	8	–	–	–	–	–	–	–	–	8
	Battagram	–	–	10	–	–	–	–	–	–	1	–	11
	Lower dir	–	–	19	–	1	–	–	–	–	–	1	21
	Mansehra	–	–	6	5	–	–	–	–	–	–	–	11
	Swat	–	–	6	3	–	–	–	1	1	–	–	11
Punjab (39)	Sialkot	12	2	1	2	–	–	1	–	–	–	–	18
	Gujranwala	6	–	–	1	–	1	–	–	–	–	–	8
	Hafizabad	1	–	1	4	–	–	–	–	–	–	–	6
	Narowal	3	–	–	2	–	–	–	–	–	–	–	5
	Sheikhupura	2	–	–	–	–	–	–	–	–	–	–	2
	Total	24	2	51	17	1	1	1	1	1	1	1	101

*Number of strains belonging to one genotype

#Total number of strains collected in this district

were screened against the *indica*/*japonica* near isogenic lines (NILs) containing different *R* genes (IR24, IRBB3, IRBB4, IRBB5, CBB23, and Kit-Xa1) normally targeted by TAL effectors. The Pakistani-*Xoo* genotypes, along with reference strains (PXO99^{A}, PXO86, and LN18), were inoculated by leaf clipping method into the field plants and the lesion length was measured at 14 dpi (Table 3; Additional file 2: Figure S4a). Plants with lesion lengths of 0–3 and 3–5 cm were considered resistant and moderately resistant, while 5–8 and > 8cm were considered as moderately susceptible and susceptible, respectively. Interestingly, all the strains were found incompatible with rice CBB23, especially KP-3, KP-24, LD-13, and BG-3, which were compatible against NILs, similar to PXO99^{A} (Table 3). In contrast, P-14, P-27, and P-35 strains were incompatible with IR24 and its tested NILs. P-18, P-10, and LD-5 strains were incompatible against IRBB5, similar to PXO86. KP-22 was the only strain found incompatible with IRBB4 and IRBB5. Rice NILs IR24, IRBB3, and Kit-Xa1 were found susceptible to all tested Pakistani *Xoo* strains (Table 3; Additional file 1: Table S3; Additional file 2: Figure S4a). Overall, virulence assays revealed that all Pakistani *Xoo* strains harbor AvrXa23, and most of them also have iTALE, which triggers *Xa23* and *Xa1*-mediated resistance and susceptibility, respectively.

Germplasm screening for TALes-dependent susceptibility

An assessment of the major virulence TALEs was performed by virulence assays on rice lines featuring polymorphisms in the *OsSWEET* promoter. The *japonica* rice variety Kitaake and its derivative lines, carrying mutations in the three *OsSWEET* genes normally targeted by TALes, were challenged with 11 Pakistani *Xoo* genotypes representing a collection of 101 strains, including well-known strains PXO99^{A}, PXO86, and LN18 for the comparative purposes (Table 3; Additional file 1: Table S3; Additional file 2: Figure S4b). Kitaake NILs, accommodating *OsSWEET11a*, *OsSWEET13*, and *OsSWEET14* mutated EBEs either singly (MS1K, MS3K, and MS4K) or in double and triple combinations (MS13K, MS14K, MS34K, and MS134K), were generated by CRISPR Cas9 (Additional file 1: Table S4). The 14 dpi lesion length measured between 0–3 and 3–5 cm was considered resistant and moderately resistant, while lengths between 5–8 and > 8cm were considered moderately susceptible and susceptible, respectively.

Three genotypes, namely P-14 (G2), P-35 (G4), and P-27 (G6), representing the diversity of the collection, were not virulent on the *japonica* reference line Kitaake. Likewise, these genotypes exhibited similar phenotypes on *indica* parental line IR24 and its NILs containing *R* genes (Table 3; Additional file 1: Table S3; Additional file 2: Figure S4). These genotypes, containing 20 of 101 strains, may be solely dependent on a novel variant of

Table 3 Pathogenicity assay of Pakistani *Xoo* genotypes in susceptibility genes edited and different *R*-genes near-isogenic rice lines

Strain/genotype	Kitaake NILs accommodating *S* genes mutated EBEs								IR24/Kitaake NILs containing different *R* genes					
	Kitaake	MS1K	MS3K	MS4K	MS13K	MS14K	MS34K	MS134K	IR24	IRBB3	IRBB4	IRBB5	CBB23	Kit-Xa1
PXO99^{A}	S	R	S	S	R	R	S	R	S	S	S	S	R	S
LN18	S	S	S	S	S	S	R	R	S	S	S	R	R	R
PXO86	S	S	S	R	S	R	R	R	S	R	S	R	R	S
P-10	S	R	S	S	R	R	S	R	S	S	S	R	R	S
P-14	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
P-18	S	S	S	R	S	R	R	R	S	S	S	R	R	S
P-27	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
P-35	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
KP-3	S	MR	S	S	MR	MR	S	R	S	S	S	MS	R	S
KP-22	S	MS	S	S	MS	MS	S	MS	S	S	MR	R	R	S
KP-24	S	R	S	S	MR	MR	S	R	S	S	S	S	R	S
LD-5	S	MS	S	S	MS	MS	S	MS	S	S	MS	MR	R	S
LD-13	S	MR	S	S	MR	MR	S	R	S	S	S	MS	R	S
BG-3	S	R	S	S	R	R	S	R	S	S	S	S	R	S

Reactions of cv. Kitaake and Kitaake with edited *OsSWEET* genes EBE (MS1K, mutation in *OsSWEET11a*; MS3K, mutation in *OsSWEET13*; MS4K, mutation in *OsSWEET14* and so on in double and triple combinations); Kit-Xa1 (Kitaake with *Xa1* gene), and near-isogenic lines (NIL) carrying different *R*-genes (IR24, IRBB3, IRBB4, IRBB5, and IRBB23) against Pakistani *Xoo* genotypes. The well-characterized strains from Philippines (PXO99^{A} and PXO86) and China (LN18) were included for reference. R, resistant (lesion length ≤ 3.0 cm); MR, moderately resistant (lesion length 3.0–5.0 cm); MS, moderately susceptible (lesion length 5.0–8.0 cm) and S, susceptible (lesion length ≥ 8.0 cm). *Xoo* strains/genotypes KP-22 and LD-5 show compatibility with cv. Kitaake and its EBE defective rice lines (italic highlighted), whereas P-14, P-27, and P-35 represent non-compatible genotypes to all cv. Kitaake and IR24 NILs (bold highlighted). The experiment was repeated and produced similar results

PthXo2-like effector (PthXo2**), i.e., PthXo2, which was incompatible with Kitaake but compatible with IR24, while PthXo2B was compatible with Kitaake and incompatible with IR24 (Oliva et al. 2019). Alternatively, cv. Kitaake and IR24 may have a resistance gene that is effective against these 20 strains.

Of the remaining 81 *Xoo* strains, one strain P-18 (G7) was not virulent on edited rice lines, which are defective in the EBE targeted by PthXo3/AvrXa7 in *OsSWEET14* (MS4K, MS14K, MS34K, and MS134K), similar to the reference strain PXO86. Genotypes P-10 (G1), KP-3 (G3), KP-24 (G9), LD-13 (G5), and BG-3 (G10), representing 78 strains, were avirulent on rice lines which are defective in the EBE of PthXo1 within *OsSWEET11a* (MS1K, MS13K, MS14K, and MS134K), similar to the reference strain PXO99^{A} (Table 3; Additional file 1: Table S3; Additional file 2: Figure S4b). Phenotypic assays revealed that none of the strains harbored PthXo1 and/ or PthXo2 and/ or PthXo3/AvrXa7, simultaneously. Moreover, the remaining two strains, KP-22 (G8) and LD-5 (G11), elicited susceptible phenotype on all edited rice lines, which harbor mutant alleles of *OsSWEET11a*, *OsSWEET13,* and *OsSWEET14*, whereas the lesion length was reduced in *OsSWEET11a* defective EBE, unlike Kitaake plants (Fig. 3a). Altogether, these findings indicate that 78 strains from the screen rely on major TALEs that target known EBE of *OsSWEET11a* and one strain on *OsSWEET14*, whereas two deviant strains, KP-22 and LD-5, have different virulence abilities, presumably possessing novel virulent TALE/s. These strains are originated from KP, and the virulence assay distinguished that these deviant strains, individually, carry both *PthXo1*-like plus another novel major virulent TALE (MVT) gene/s (Table 3; Fig. 3a; Additional file 1: Table S3; Additional file 2: Figure S4b).

Characterization of deviant strains

To further test whether the novel TALE/s possibly target/s the new EBE for disease susceptibility, we conducted expression analysis of *OsSWEET11a*, *OsSWEET11b*, *OsSWEET12*, *OsSWEET13*, *OsSWEET14*, and *OsSWEET15* by inoculating ddw (double-distilled water), KP-22, LD-5, and LN18 into Kitaake and MS134K plants (Fig. 3b), with ddw and *Actin* served as external and internal controls, respectively. LN18 was the sole representative strain harboring PthXo2 like (Tal5) and AvrXa7 effectors targeting both *OsSWEET13* and *OsSWEET14*. *OsSWEET13* and *OsSWEET14* were significantly expressed in Kitaake but not in MS134K plants infected with LN18. This finding was consistent with previous studies where Tal5 and AvrXa7 were shown to be the major virulent TALEs in LN18 (Xu et al. 2019). Similarly, only *OsSWEET11a* was significantly expressed in Kitaake rice infected with LD-5 and KP-22 but not in MS134K, and the *OsSWEET11a* defective EBE plants were moderately susceptible compared with Kitaake (Fig. 3). On the other hand, *OsSWEET*s edited plants were moderately resistant or resistant to all other Pakistani *Xoo* genotypes. These results suggest that LD-5 and KP-22 contain two major TALEs, i.e., PthXo1-like plus another novel MVTs that contribute to the susceptibility of defective EBE plants. Moreover, LD-5 and KP-22 could not induce the expression of *OsSWEET11b*, *OsSWEET12*, and *OsSWEET15*, indicating the presence of unidentified susceptibility gene/s other than the tested *OsSWEET* genes.

Deployment of rice varieties based on major TALEs prediction in Pakistani-*Xoo* strains

To identify the major TALEs in Pakistani-*Xoo* strains, we used cv. IR24 and Kitaake NILs containing genes targeted by TALEs. The major TALEs were inferred by analyzing the phenotypic data obtained from field inoculations. Notably, rice lines in the background of IR24/Kit, i.e., IRBB5, CBB23, and Kit-Xa1 carry *xa5*, *Xa23*, and *Xa1*, which are targeted by PthXo7, AvrXa23, and iTALE, respectively. Briefly, *Xoo* strains containing PthXo7 target the recessive *R* gene *xa5*, leading to a susceptible phenotype, while strains that harbor iTALE suppress the *Xa1*-dependent resistance. Moreover, *Xa23* is the executor *R* gene and confers broad-spectrum resistance to *Xoo* strains harboring AvrXa23 (Hutin et al. 2015; Ji et al. 2022). Additionally, to estimate the major TALEs targeting susceptibility genes, we used CRISPR-Cas9 edited rice lines containing mutations in all three EBEs recognized by the major TALEs, i.e., PthXo1/PthXo1* (Tal6b), PthXo2*, and PthXo3/AvrXa7 (Liu et al. 2024). These findings provided valuable guidance for the strategic deployment of disease-resistant rice varieties. For instance, to identify suitable rice varieties resistant to bacterial blight across various regions of Pakistan, we analyzed the geographic distribution of 101 *Xoo* strains and their probable *R* and *S* genes. Based on the phenotypes, all *Xoo*-strains harbor AvrXa23, while 81, 54, and 20 strains contain iTALE, PthXo7, and PthXo2**, respectively (Fig. 4). For susceptibility genes, 78 *Xoo* strains possessed major TALEs that could potentially activate *OsSWEET11a*. There was one *Xoo* strain that could possibly activate *OsSWEET14*. Two KP (Swat and Lowe dir) *Xoo* strains activated *OsSWEET11a* along with unidentified susceptibility gene/s (Figs. 3, 4). Most strains originating from Punjab activated *OsSWEET11a* and *OsSWEET14*, while strains from KP activate *OsSWEET11a* but not *OsSWEET13* or *OsSWEET14*. Our results suggest that EBE editing of *OsSWEET11a* and *OsSWEET14* could be employed to mitigate BB effectively in Punjab.

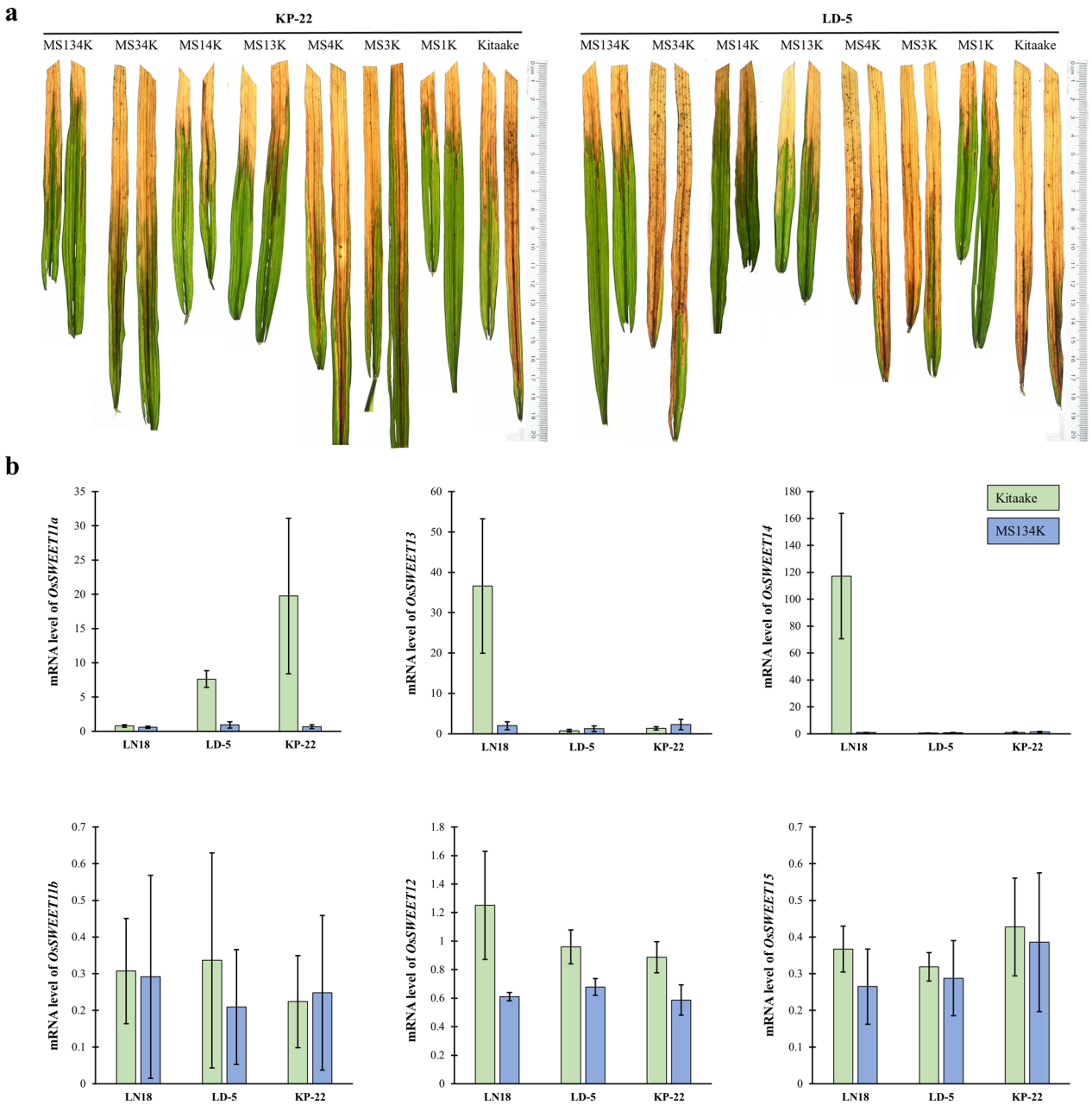

Fig. 3 Virulence and *OsSWEETs* expression induced by KP-22 and LD-5 in Kitaake and MS134K rice lines. **a** Lesion length induced by KP-22 and LD-5 in cv. Kitaake and major TALEs EBE edited rice lines. Bacterial strains were inoculated by tip-cutting and images were captured 14 days after inoculation. The figures display representative disease lesions, and the mean lesion lengths with standard deviation (n = 8) are detailed in Additional file 1: Table S3. **b** mRNA levels of *OsSWEET11a*, *OsSWEET11b*, *OsSWEET12*, *OsSWEET13*, *OsSWEET14*, and *OsSWEET15* ($2^{-\Delta\Delta Ct}$) determined by qRT-PCR in cv. Kitaake and MS134K leaves treated with double-distilled water (ddw, mock inoculation), LN18, LD-5, and KP-22 (compatible strains to MS134K) at 24 hpi. The strain LN18 was used for reference, ddw as external control and rice *Actin*-6 gene as an internal control. Data represent replicated (n = 3) qRT-PCR

To illustrate the relatedness of Pakistani-*Xoo* strains, a dendrogram was generated considering the presumed major TALEs based on the virulence assessment of NILs containing *R*/*S* genes using NTSYSpc-2.02e. The *Xoo* genotypes plus reference strains were clustered into 6-groups (Fig. 4). The estimated virulence/avirulence effectors carried by the genotype and their cognate genes in NILs are shown in the table (Fig. 4). Interestingly, these results are in congruence with the single available genome of Pakistani *Xoo* strain PkXoo1 (Additional file 1:

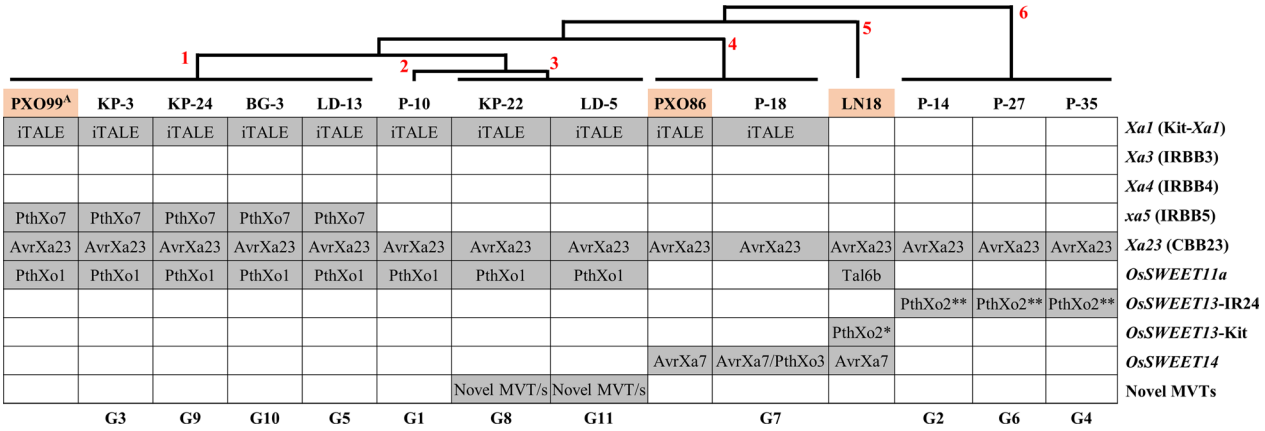

Fig. 4 Major TALEs prediction in Pakistani-*Xoo* strains. The assignment of the major TALEs was based on the virulence assessment of *Xoo* strains upon inoculation into Near-Isogenic Lines (NILs) harboring resistance genes or edited susceptibility genes in cv. IR24 and Kitaake, defining the relative contributions of multiple avirulence/virulence effectors. At the top, parsimony tree was generated using the NTSYSpc-2.02e, and all the *Xoo* genotypes including reference strains were clustered into 6 groups (1–6 in red fonts). The estimated avirulence/virulence TAL effectors carried by the genotypes/strains are shown in the table. The representative reference strains are orange highlighted and genotypes are shown at the bottom

Table S5). Collectively, our findings suggest that CBB23 is the most effective rice line that could be used to combat *Xoo* in Pakistan, especially in Punjab and Khyber Pakhtunkhwa.

Discussion

Transcription activator-like effectors (TALEs), recognized as virulence markers of *Xanthomonas* spp., are highly evolvable and subject to selective pressures, playing crucial roles in various plant diseases. In this study, we aimed to describe the population structure of the Pakistani *Xoo* collection through the analysis of TALEs diversity and aggressiveness of the strains on Kitaake NILs with defective EBEs/IR24 NILs containing *R* genes. In total, 101 *Xoo* strains were collected from the two rice-growing provinces in Pakistan (Additional file 2: Figure S1) and screened via PCR using pathovar-specific primers (Additional file 2: Figure S2). TALE-based genotyping clustered the 101 isolates into 11 genotypes (Fig. 1; Additional file 2: Figure S3) containing 16 to 20 potential TALE fragments (Table 1). Amongst, 51, 24, and 17 strains were assigned to genotypes 3, 1, and 4, respectively (Table 1). TALE diversity analysis further categorized the 11 genotypes into 5 clades. Notably, the clade pattern aligned with the geographic distribution of the strains except for G3 and G4 encompassing strains from both provinces and clade 4 of G7 and G9 (Fig. 2). Virulence assays on NILs containing different *R* genes revealed that CBB23 is the most effective rice line against all Pakistani *Xoo* genotypes (Table 3; Additional file 1: Table S3; Additional file 2: Figure S4a). Moreover, pathogenicity assays on NILs containing defective EBEs for the three major virulent TALEs (PthXo1, PthXo2, and PthXo3/AvrXa7) revealed two deviant strains, KP-22 and LD-5, harboring PthXo1-like plus novel MVTs contributing to susceptibility (Table 3; Additional file 1: Table S3; Additional file 2: Figure S4b). Intriguingly, expression analysis of six clade-III *OsSWEET* genes suggests the presence of novel TALE/s targeting previously unidentified susceptibility gene/s (Fig. 3). These findings provide valuable insights into the diversity and pathogenicity of the Pakistani *Xoo* population and highlight the complex interactions between TALEs and host plants (Fig. 4).

Bacterial blight (BB) was first reported in Pakistan in the mid-1970s, and recent surveys conducted in major rice-growing areas of three provinces—Punjab, Sindh, and Khyber Pakhtunkhwa —have shown a continuous increase in disease incidence. The prevalence of BB is particularly notable in Punjab and KP, as reported in a study by Ahsan et al. (2021). In response to this concerning trend, we sought to provide a comprehensive understanding of the TALome diversity and aggressiveness of *Xoo* isolates collected from these two provinces (Additional file 2: Figure. S1). To date, only one complete genome sequence of a Pakistani *Xoo* strain, PkXoo1, has been sequenced (Ejaz et al. 2023). Through TALE prediction and analysis, we discovered that PkXoo1 harbors 18 *tal* genes, a finding consistent with our Southern blot results, which indicated the presence of 16, 17, 18, and 20 TALE fragments (Table 1). Similarly, genome analysis of 33 Asian *Xoo* strains, whether derived from databases or newly sequenced, demonstrated the presence of 18–21 TALE loci per genome (Oliva et al. 2019). Notably, Pakistani *Xoo* strains exhibit a remarkably high genetic

diversity in terms of TALEs, surpassing the extent that has been previously elucidated. This heightened genetic diversity in the TALome of Pakistani *Xoo* strains may have implications for the increased incidence and prevalence of bacterial blight in the regions, particularly in Punjab and KP.

The impact of TAL effectors on pathogen virulence or avirulence can vary significantly, ranging from major to moderate or even undetectable. In this study, our focus was on identifying TAL effectors within Pakistani *Xoo* strains that might function as avirulence (avr) or virulence factors. Utilizing varieties carrying resistance genes is considered a highly effective method for controlling BB in rice. Dominant resistance is often triggered by TALE-mediated induction of executor genes, whose expression leads to rapid plant cell death, thereby impeding disease development (Boch et al. 2014). Here, toward identifying sources of resistance effective against Pakistani *Xoo* strains, we assessed the putative avr activity of 11 genotypes (representing the collection of 101 strains) on rice accessions possessing *Xa1*, *Xa3*, *Xa4*, *xa5*, and *Xa23* genes. The rice line containing *Xa23* was most effective against Pakistani *Xoo* strains, followed by *xa5* and *Xa4* (Table 3; Fig. 4; Additional file 1: Table S3; Additional file 2: Figure S4a). TALEs comparative analysis revealed that PkXoo1 harbors PthXo1, PthXo6, PthXo7, PthXo8, AvrXa23, and AvrXa27 (with 0–5 RVDs differences) (Additional file 1: Table S5). This observation was consistent with the phenotypes, i.e., *Xa23* comes up as one of the most promising *R* genes in terms of resistance spectrum for the Pakistani *Xoo* population. In conclusion, *Xa23* emerges as an excellent material for breeding to develop improved rice varieties with enhanced resistance to the conditions in Pakistan.

Numerous reports indicate that *tal* genes play a significant role in virulence across various *Xanthomonas* species, targeting the EBEs in the promoter region of susceptibility genes (Yang et al. 2006; Antony et al. 2010; Yu et al. 2011; Streubel et al. 2013; Zhou et al. 2015; Tran et al. 2018; Xu et al. 2019; Xu et al. 2023). Mutation of these EBEs is being employed as a pivotal strategy to develop resistant crops against TALE-dependent pathogens (Eom et al. 2019; Oliva et al. 2019; Xu et al. 2019). However, a potential obstacle to the successful implementation of this strategy arises from TALE adaptations through the rearrangement of their repeat regions (Teper and Wang 2021). The current investigation involved the assessment of various *Xoo* genotypes from Pakistan on different rice lines, with single, double, and triple edits of major TALEs EBE sequences in the promoters of three *OsSWEET* genes in the rice cv. Kitaake. This unique set of rice materials proved effective in predicting major virulent TALEs in *Xoo* strains through plant inoculation and phenotypic monitoring, without relying on genomic sequencing. The effectiveness of this set of materials was verified through genomic prediction and expression of susceptibility genes (Liu et al. 2024). The assortment of *Xoo* strains from diverse geographical regions, their inoculation onto edited rice lines, and the utilization of the TALE prediction table offer valuable insights for more informed breeding strategies to enhance bacterial blight resistance in rice (Liu et al. 2024). In screening 11 Pakistani *Xoo* genotypes through inoculation tests, three genotypes, representing 20 strains, failed to induce disease on Kitaake and its edited rice lines. This failure was attributed to the presence of only PthXo2-like TAL effectors in these strains, which are incompatible with the *OsSWEET13* EBE of Kitaake. Amongst, five genotypes representing 78 strains unable to elicit susceptibility on *OsSWEET11a* EBE defective rice lines (MS1K, MS13K, MS14K, and MS134K), suggesting the presence of PthXo1 in these strains. *OsSWEET14* EBE edited rice lines (MS4K, MS14K, and MS134K) displayed resistance to only one genotype, indicating the presence of PthXo3/AvrXa7 TAL effector. Notably, none of the genotypes contained two or three major TAL effectors simultaneously. Moreover, two genotypes (KP-22 and LD-5) were capable of inducing a susceptible phenotype on cv. Kitaake and all seven edited lines (reduced lesion length on *OsSWEET11a* EBE edited rice lines), implying the emergence of a new virulence TALE/s that can induce unidentified susceptibility along with PthXo1/like. These strains harbored the highest number of *tal* genes, specifically 20. Additionally, an expression analysis of *OsSWEET* genes was conducted to determine whether the novel TALE/s targeted a novel EBE among the major susceptibility genes (Figs. 3, 4). Altogether, these results highlight an important *tal* gene diversity of the *Xoo* population from Pakistan.

Bacterial blight, caused by *Xoo*, exhibits the potential for multiple *Xoo* races to incite the disease within a given geographical area. Regional convergence is evident among *Xoo* strains, showcasing notable diversity in races across different geographical regions. For instance, in the central-eastern (Punjab) and northwestern (Khyber Pakhtunkhwa) rice cultivation areas of Pakistan, nearly all *Xoo* strains carry PthXo1 which can activate *OsSWEET11a*. Notably, a single strain from the central-eastern region possesses PthXo3/AvrXa7, while two strains from the northwestern region can activate both *OsSWEET11a* and unidentified susceptibility gene/s simultaneously, as observed in *Xoo* KP-22 and LD-5. A parallel study of northeastern China reveals that most *Xoo* strains activate *OsSWEET13* and *OsSWEET14*; some strains can concurrently activate *OsSWEET11a*, *OsSWEET13*, and *OsSWEET14*, exemplified by *Xoo* LN4, LN2, and LN18

(Liu et al. 2024). The regional differentiation is more prominent in the vast rice cultivation region in southern China, where the majority of strains selectively target *OsSWEET14* (Liu et al. 2024). In environments with low selection pressure, favorable conditions facilitate the development of an initial inoculum load, leading to plant infections. Additionally, genetic flow plays a crucial role in the dissemination of pathogens among diverse field populations. These findings inspire innovative approaches in rice cultivation, such as developing cultivars with multiple mutations in EBEs, potentially conferring broad-spectrum resistance. Moreover, considering the expression patterns of *OsSWEET11a*, *OsSWEET13*, and *OsSWEET14* in response to *Xoo*, coupled with geographical origin, offers insights into identifying effective editing variants for optimizing rice cultivation practices (Eom et al. 2019).

Although it is tempting to associate the presence of a specific band with virulence, such an association remains highly speculative. Therefore, a more valuable approach involves isolating or engineering an *Xoo* strain devoid of TALEs. This strain could then be utilized to reintroduce TALE genes systematically, allowing for the thorough dissection of the individual contributions of each TALE gene to virulence (Shah et al. 2019; Haq et al. 2020). Molecular identification of TALE-induced target genes in host plants becomes imperative for a comprehensive understanding of the molecular mechanisms underlying TALE-promoted diseases (Boch et al. 2014). The substantial progress in TALE biology has significantly enhanced our comprehension of the interplay between TALEs and their host targets. This progress has paved the way for the development of innovative methods for identifying TALE-targeted EBEs, including TALE-code-based EBE prediction, β-glucuronidase assay, electrophoretic mobility shift assay, and designer TALEs (Antony et al. 2010; Li et al. 2013; Haq et al. 2021; Shah et al. 2023).

In this study, we provided insights into the TALome diversity and aggressiveness of the Pakistani *Xoo* population. Our findings aim to bridge existing gaps in understanding whether TALE diversity is primarily linked to geographic regions. Notably, we identified that the rice line containing *Xa23* is the most effective variety for breeding in Pakistan. Furthermore, genotypes G8 and G11, represented by KP-22 and LD-5, respectively, harbor an unknown virulence factor/s distinct from other tested *Xoo* genotypes/strains. These genotypes can overcome the resistance conferred by the MS134K rice variety, known for its broad-spectrum resistance to BB (Eom et al. 2019; Oliva et al. 2019; Xu et al. 2019; Liu et al. 2024). This suggests that KP-22 and LD-5 may represent emerging minority strains that have naturally co-evolved effectors to challenge the resistance pressure in rice fields. In general, strains with a higher number of putative TAL effector genes exhibited increased virulence compared to strains with fewer TAL genes. Additionally, expression analysis revealed co-evolved alleles targeting unidentified susceptibility gene/s that contribute to disease development. Overall, our study highlights the existence of gene-for-gene relationships between the tested rice lines and Pakistani *Xoo* strains. This marks the initial report presenting the diversity of TALEs and their association with *R* and *S* genes. Further efforts to identify novel virulent TALE/s and their target/s in cv. Kitaake is imperative to precisely elucidate their role in BB.

Conclusions

This study provides the first comprehensive analysis of the TALome diversity in the Pakistani *Xoo* population and its association with rice *R* and *S* genes. Pakistani *Xoo* strains exhibited extensive TALE diversity, with 16–20 putative TALE fragments and 11 distinct genotypes classified into five clades, reflecting geographic distribution with some exceptions. Notably, genotypes G8 (strain KP-22) and G11 (strain LD-5) were identified as harboring novel virulent TALEs that target previously unidentified susceptibility genes, overcoming broad-spectrum resistance in MS134K rice. Virulence assays revealed the effectiveness of *Xa23*-containing rice lines against all tested strains, emphasizing its potential for breeding in Pakistan. However, the emergence of strains capable of circumventing widely deployed resistance genes highlights the need for continued surveillance and functional characterization of novel TALEs. These findings underscore the gene-for-gene relationships between Pakistani *Xoo* strains and rice lines, emphasizing the necessity of identifying virulent TALEs and their targets to refine resistance breeding strategies and ensure sustainable management of bacterial blight.

Methods

Bacterial strains, and growth conditions

Bacterial strains used in this research are listed in Additional file 1: Table S1. *Xoo* strains were cultured in nutrient broth (NB; 5 g polypeptone, 10 g sucrose, 1 g yeast extract, and 3 g beef extract in one liter of distilled water, pH = 7.0–7.2) or NB with 1.5% agar (NA) at 28°C. Antibiotics were used at the following concentrations (μg/mL) when required: ampicillin (Ap) 100 μg/mL and spectinomycin (Sp) 40 μg/mL.

Bacterial strains collection

Surveys to collect the bacterial blight disease specimens were conducted between September and November of 2017, 2018, 2019, and 2021 in the key rice production regions of two provinces of Pakistan. In Punjab (P)

province; Hafizabad, Sheikhupura, Sialkot, Narowal, and Gujranwala districts were surveyed (Additional file 2: Figure S1). In Khyber Pakhtunkhwa (KP) province; surveys were conducted in Mansehra, Battagram, Bannu, Swat, and Lower Dir districts (Additional file 2: Figure S1). Disease leaf samples were collected based on the presence of typical BB symptoms from cultivated rice varieties.

For the isolation of *Xoo*, lesion segments were subjected to disinfection using 70% ethanol and were subsequently rinsed three times with sterilized distilled water. The samples were then cut into small pieces using sterilized scissors and immersed in double distilled water for one hour at room temperature to facilitate the release of bacteria. The resulting suspension was then streaked onto fresh plates of NA followed by incubation at 28°C for a period of 5–7 days. Single pure colonies were screened molecularly through PCR using pathovar-specific primers (Xoo80F/Xoo80R) (Lang et al. 2010). Validated *Xoo* strains were preserved in 50% glycerol at − 80°C. A total of 101 Pakistani *Xoo* strains were used in this study, along with three reference strains, two from Philippines ($PXO99^A$ and PXO86), and one from China (LN18).

Southern blot hybridization

The genomic DNA was extracted using the HiPure Bacterial-DNA extraction Kit (Magen, Guangdong, Guangzhou, China) following the manufacturer's protocols. The quality and quantity of the isolated DNA were checked with NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, USA). An aliquot of 10 µg of DNA was digested with *Bam*HI (3 µg/µL) restriction endonuclease (Takara, Japan) and incubated at 37°C for 5 h. The digested DNA was separated on 1.3% agarose gel in 1 × TAE buffer (50 × = Tris 242 g, $Na_2EDTA{\cdot}2H_2O$ 37.2 g, and 57.1 mL CH_3COOH in one liter distilled water, pH = 8.5) at 80 V, 4°C for 20 h. Subsequently, the gel was kept in denaturation buffer (0.5 M NaOH and 1.5 M NaCl) and neutralization buffer (0.5 M Tris–HCl and 1.5 M NaCl, pH = 7.5), shaking for 45 and 15 (twice) minutes, respectively. The DNA fragments were then blotted from the gel on a presoaked Hybond N^+ nylon membrane (Millipore, Billerica, MA, USA) using standard saline citrate buffer (tri-sodium citrate di-hydrate 88.2 g and NaCl 175.3 g, pH = 7.0) as capillary transfer solvent. The *Xoo* strain $PXO99^A$ *PthXo1*, i.e., pZY-*PthXo1* (Additional file 1: Table S1) internal *Sph*I fragment (2892 bp; containing 24 RVDs) was labeled with digoxigenin (DIG) and employed as a hybridization probe for the detection of TALE fragments. Hybridization was done by adding the labeled probe into the DIG Easy hybridization buffer and incubating it in a rotatory hybridization machine at 68°C overnight. Probe labeling, hybridization, blocking, and detection were conducted following the manufacturer's instructions (Roche Diagnostics GmbH Mannheim, Germany). The *Xoo* $PXO99^A$ TALE-free strain PH harboring pHZY-*PthXo1*, pHXY-*PthXo2*, and pHZY-*AvrXa7 in trans* were used to point out the major virulence *tal* genes (Additional file 1: Table S1).

Genetic similarity based on TALE repertoires was computed using the NTSYSpc-2.02e. The number and size of putative TALEs were converted into binary form, where a value of 1 denotes the presence of a specific band, and 0 indicates the absence of the corresponding band. The results were subjected to generating the dendrogram using the UPGMA method within the SimQual similarity and Sequential Agglomerative Hierarchical Nested (SAHN) clustering module of NTSYS 2.02e as described by Jamshidi and Jamshidi (2011).

Pathogenicity analysis

Rice seeds were soaked in water and incubated at 37°C for 48–72 h. The seedlings were then transferred into the greenhouse and, after 2–3 weeks, planted in the field. *Oryza sativa* subsp. *indica*/*japonica* containing different *R* genes (IR24, IRBB3, IRBB4, IRBB5, CBB23, and Kit-Xa1) and *Oryza sativa* subsp. *japonica* containing EBE mutants of *OsSWEET11a*, *OsSWEET13*, and *OsSWEET14* [cv. Kitaake, MS1K (*OsSWEET11a* mutant), MS3K (*OsSWEET13* mutant), MS4K (*OsSWEET14* mutant), MS13K (*OsSWEET11a* and *OsSWEET13* mutant), MS14K (*OsSWEET11a* and *OsSWEET14* mutant), MS34K (*OsSWEET13* and *OsSWEET14* mutant), and MS134K (*OsSWEET11a*, *OsSWEET13,* and *OsSWEET14* mutant)] were grown in the field conditions at Shanghai Jiao Tong University, Shanghai, China.

To investigate whether there are rice lines resistant to Pakistani *Xoo* strains, 14 NILs containing different *R* genes and *S* genes-edited rice lines were used in the virulence assessment of the collected Pakistani strains. *Xoo* strains grown overnight were employed to infect two-month-old rice plants (at the booting stage) using the tip-cutting method. Disease symptoms were assessed 14 days post-inoculation (dpi), and the lesion length (cm) was recorded. More than five flag leaves were inoculated with each *Xoo* strain, and experiments were repeated twice. Standard deviation analyses were performed on all measurements. Strain aggressiveness and disease severity were evaluated based on lesion length index as follows: measurements 0–3 cm were scored as resistant (R), 3–5 cm as moderately resistant (MR), 5–8 cm as moderately susceptible (MS), and > 8 cm as susceptible (S).

Confirmation of Kitaake mutant rice lines

The three major susceptibility genes' EBE mutant plants (single, double, and triple mutants) in the background of cv. Kitaake were generated by Liu et al. (2024) for tracing

the major virulence TAL effectors in *Xoo* strains. To confirm the EBE-edited plants, we isolated the genomic DNA from all the mutants using the CTAB (cetyltrimethylammonium bromide) method. The *OsSWEET11a*, *OsSWEET13*, and *OsSWEET14* promoter regions harboring EBEs were PCR amplified using primer sets SW11p-F/SW11p-R, SW13p-F/SW13p-R, and SW14p-F/SW14p-R, respectively (Additional file 1: Table S2). The PCR amplicons were sequenced by Sanger sequencing and comparatively analyzed (Additional file 1: Table S4).

Expression analysis of six *OsSWEET* genes

The indicated bacterial strains (washed twice and resuspended in double distilled water, $OD_{600}=0.6$) were inoculated into four-week-old rice seedlings (grown in the greenhouse with a photoperiod of 14 h light and 10 h of dark at 25°C) using a needleless syringe, and samples were collected at 24 h after inoculation. Total RNA was extracted using RNAiso Plus (TAKARA BIO INC., Japan) reagent according to the manufacturer's protocol. The quality and quantity were assessed using NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, USA). An aliquot of 1 µg RNA from each sample was reverse transcribed into cDNA using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech Beijing, China). The qRT-PCR was performed on Applied Biosystems 7500 Real-Time PCR System using TransStart Tip Green qPCR Supermix (+DyeII) Kit (TransGen Biotech Co., Ltd. Beijing, China). The PCR conditions were 30 s at 95°C, followed by 40 cycles at 95°C, 60°C, and 72°C for 10 s, 34 s, and 15 s, respectively. The relative expression levels of *OsSWEET11a*, *OsSWEET11b*, *OsSWEET12*, *OsSWEET13*, *OsSWEET14*, and *OsSWEET15* were calculated with the $2^{-\Delta\Delta CT}$ method and normalized to the expression of rice *Actin* gene. The primers used in qRT-PCR are listed in Additional file 1: Table S2.

Statistical analysis

The genomic sequence of the single available Pakistani *Xoo* strain, PkXoo1 (Genbank accession no. CP101721.2), was retrieved from the National Center for Biotechnology Information. TALEs were annotated and analyzed using AnnoTALE v1.2 (Grau et al. 2016). The *Bam*HI fragments of *tale* sequences were simulated in gel using SnapGene 6.0.2 and compared with the Southern blot results to understand the intensity of bands and possible RVDs of TAL effectors (data not shown). Strains clustering based on the number and size of TALEs and major virulent TALEs were conducted using the UPGMA method in NTSYSpc-2.02e, as described earlier.

Abbreviations

BB	Bacterial blight
Xoo	*Xanthomonas oryzae* Pv. *oryzae*
TALEs	Transcription activator like effectors
S gene	Susceptibility gene
R gene	Resistance gene
CBB	China bacterial blight
IRBB	International rice bacterial blight
EBEs	Effector binding elements
NILs	Near-isogenic lines
OsSWEET	*Oryza sativa* Sugar will eventually exported transporter
T3SS	Type-III secretion system
T3Es	Type-III effectors
CRR	Central repeat region
RVD	Repeat variable di-residue
NLS	Nuclear localization signal
AD	Activation domain
truncTALEs	Truncated TALEs
iTALEs	Interfering TALEs
KP	Khyber Pakhtunkhwa
P	Punjab
LD	Lowe dir
BN	Bannu
BG	Battagram
MS1K	Mutant *OsSWEET11a* Kitaake
MS3K	Mutant *OsSWEET13* Kitaake
MS4K	Mutant *OsSWEET14* Kitaake
MS13K	Mutant *OsSWEET11a* and *OsSWEET13* Kitaake
MS14K	Mutant *OsSWEET11a* and *OsSWEET14* Kitaake
MS34K	Mutant *OsSWEET13* and *OsSWEET14* Kitaake
MS134K	Mutant *OsSWEET11a*, *OsSWEET13* and *OsSWEET14* Kitaake
qRT-PCR	Quantitative real-time polymerase chain reaction
SB	Southern blot
G	Genotype
MVT	Major virulent TALE
NXO	Pakistan *Xanthomonas oryzae*

Supplementary Information

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Additional file 1: Table S1. Bacterial strains and plasmids used in this study. **Table S2.** Primers used in this study. **Table S3.** Disease score of Xoo strains on rice cv. IR24 and Kitaake NILs. **Table S4.** EBE sequences of the three *OsSWEET* genes of rice cv. IR24, Kitaake and its mutants. **Table S5.** Pakistani strain PkXoo1 TALEs RVDs in comparison with major TALEs.

Additional file 2: Figure S1. Geographic distribution per site and per year of a collection of 101 strains of *Xanthomonas oryzae* pv. *oryzae* from Pakistan. A collection of 101 strains from two provinces (39 from Punjab and 62 from Khyber Pakhtunkhwa) of Pakistan were used in this study. All the strains were isolated from leaves collected in 2017 (n = 40), 2018 (n = 22), 2019 (n = 24), and 2021 (n = 15). Sampled districts are block shaded, and the number of strains plus collection year is denoted with pie charts. The map was generated via QGIS, representing provincial boundaries in bold black lines. **Figure S2.** PCR-based validation of Pakistani Xoo isolates. A total of 101 Pakistani Xoo strains were screened using a specific set of primers; Xoo80F/Xoo80R amplifies a 162 bp fragment. PXO99A was used as positive control, and PCR without template DNA as negative (-) control. All strains were isolated from the rice-producing areas in two provinces; Punjab and Khyber Pakhtunkhwa of Pakistan from 2017–2019 and 2021. Strain names are indicated at the top; Lane M represents marker size in base pairs (bp). **Figure S3.** Southern blotting of 101 Pakistani Xoo isolates. The genomic DNA was subjected to BamHI digestion, transferred onto nylon membranes, and subsequently probed with the SphI fragment from PthXo1. Hybridization results unveiled distinct genotypes, denoted as G1 to G11, corresponding to the respective strains indicated at the top. The strains $PXO99^{A}$ and LN18 were used for reference. λEcoT14 marker is shown in the left lane in base pairs (bp). **Figure S4.** Inoculation

phenotypes of Pakistani Xoo genotypes on cv. IR24 and Kitaake NILs. **a** Disease phenotypes of NILs containing different R genes and, Kitaake and **b** its EBE defective rice lines after inoculation with Pakistani Xoo genotypes. These phenotypes depict the functional relationship between TAL effectors and their targeted resistance/susceptibility genes. PXO99A, PXO86, and LN18 were used as reference strains. Photos were taken 14 days post-inoculation. Representative disease lesions are shown in the figure and mean lesion lengths ± SD (n = 8) are provided in Additional file 1: Table S3. Rice lines and the respective strain names are mentioned at the top of each figure. Phenotypes of CBB23, IRBB5, and IRBB4 show resistance to 101, 27, and 1 strain, respectively. Whereas phenotypes of cv. Kitaake and its EBE defective rice lines revealed that the majority of Pakistani *Xoo* stains harbor PthXo1-like effector (78 strains), one strain carries PthXo3/AvrXa7 like effector, and 20 strains contain a variant of PthXo2 like effector (PthXo2**).

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Author contributions
SS performed the experiments and RA isolated the strains. SS, RA, LL, YL, QW, JY, and YW analyzed the data. MK, XX, ZX, AR, and MZ contributed materials. SS, LL, XX, ZX, MZ, and GC planned and designed the research. SS and GC wrote an initial version of the manuscript that was read and revised by all authors.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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