Original Article

A varied AvrXa23-like TALE enables the bacterial blight pathogen to avoid being trapped by *Xa23* resistance gene in rice

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HIGHLIGHTS

- This study, for the first time, uncover a naturally-emerging *Xa23*-breaking *Xoo* isolate.
- The ability of AvrXa23 to be trapped by the EBE of *Xa23* gene can be altered by one or more RVDs of AvrXa23.
- Seven AvrXa23-like TALEs determine the "arms-race" in the AvrXa23 and *Xa23* pair.
- This study provide new insights into the diversified strategies used by *Xoo* to evade host resistance.
- Planting single *R* gene (like *Xa23*) rice in a great region is risk to make *Xoo* minority to majority.

GRAPHICAL ABSTRACT
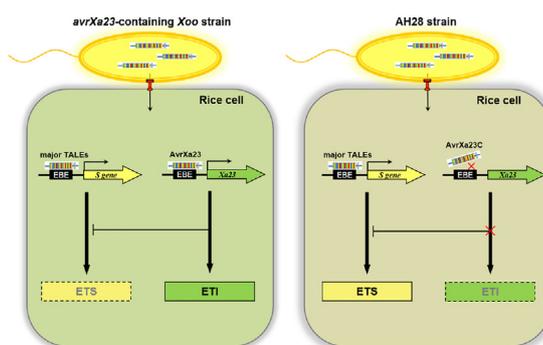
ARTICLE INFO

ABSTRACT

Introduction: Xa23 as an executor mediates broad-spectrum resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which contains a matching avirulence gene *avrXa23*, in rice for bacterial leaf blight (BLB). *avrXa23* encodes a transcription activator-like effector (TALE) protein which binds to the EBE (effector-binding element) of the *Xa23* promoter. It is unclear whether the considerable pressure of *Xa23* leads to an emerging *Xoo* strain that overcomes *Xa23* resistance.

Objectives: This study aimed to uncover new *Xoo* isolate(s) that overcome *Xa23*-mediated resistance and to investigate how the pathogen evades the resistance.

Methods: Totally 185 *Xoo* isolates were used to screen possibly compatible strain(s) with *Xa23*-containing rice CBB23 by pathogenicity test. Genome Sequencing, Southern blot, *tal* gene cloning, Western blot, qRT-PCR and electrophoretic mobility shift assays (EMSA) were conducted to determine the mechanism of one *Xoo* isolate being compatible with *Xa23*-containing rice.

Results: One isolate AH28 from Anhui province is compatible with CBB23. AH28 strain contains an ortholog of *avrXa23*, *tal7b* and has 17 *tal* genes. The 4th RVD (repeat-variable diresidue) in Tal7b are missed and the 5th and 8th RVDs changed from NG and NS to NS and S*, respectively. These alternations made Tal7b unable to bind to the EBE of *Xa23* promoter to activate the expression of *Xa23* in rice. The ectopic expression of *tal7b* in a *tal*-free mutant PH of PXO99[A] did not alter the virulence of the strain PH, whereas *avrXa23* made AH28 from compatibility to incompatibility with *Xa23* rice.

Conclusion: Best to our knowledge, this is the first insight of a naturally-emerging *Xoo* isolate that overcomes the broad-spectrum resistance of *Xa23* by the variable AvrXa23-like TALE Tal7b. The RVD alteration in AvrXa23 may be a common strategy for the pathogen evolution to avoid being "trapped" by the executor *R* gene.

Introduction

As one of the most devastating rice diseases worldwide, bacterial leaf blight (BLB) is largely modulated by a gene-for-gene manner between the pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and the host plant rice [1,2]. *Xoo* is able to successfully colonize and infect rice in the absence of resistance (*R*) genes or when *R* genes are not activated [3]. The *Xoo*-rice pathosystem has followed a 'zigzag', co-evolving arms-race competition. The outcome of this interaction is largely dependent on the effector proteins translocated into host cells by the *Xoo*-encoded type-III secretion system (T3SS) and their interaction with *R* gene products that are recognized in a cultivar/race-specific manner [3-5].

Approximately 46 genes conferring resistance to various *Xoo* races have been identified in cultivated and wild rice and artificial mutants [6,7]. At least 17 of these *R* genes have been cloned and characterized, including *Xa1, Xo1, Xa2, Xa3/Xa26, Xa4, xa5, Xa7, Xa10, xa13, Xa14, Xa21, Xa23, xa25, Xa27, Xa31(t), xa41* and *Xa45 (t)* [6,8-11]. The *R* gene *Xa23* was originally derived from wild rice (*Oryza rufipogon*) and confers dominant, broad-spectrum resistance to BLB [12,13]. *Xa23* is an executor *R* gene (*E* gene) that encodes a 113 amino acid protein (Xa23) [14]. The near-isogenic line CBB23 was obtained by transferring *Xa23* from wild rice into the susceptible *Oryza indica* cultivar JG30 [13]. The improved cultivar CBB23 exhibits broad-spectrum resistance to *Xoo*, which largely depends on the transcriptional activation of *Xa23* by effector AvrXa23 that is considered to be encoded by virtually all *Xoo* strains in the past [13,14], but skeptically in the future.

Xoo may evolve decoys or new effectors to evade *R* gene recognition and suppress the resistance triggered by effectors (ETI), resulting in effector-induced susceptibility (ETS) [3,15,16]. Among effectors, transcription activator-like effectors (TALEs) form a particular family of proteins in *Xoo* that bind effector-binding elements (EBE) in the promoter regions of plant genes encoding for resistance (*R*) or susceptibility (*S*) [17-19]. Therefore, TALEs are commonly referred to as avirulence or virulence proteins based on whether the targets are *R* or *S* genes [15,20,21]. The ability to recognize and bind EBE is due to the conserved architecture in TALEs which includes the typical components: (1) a highly conserved N-terminal region required for type III secretion; (2) a central repeat region containing a variable number of mostly 34-amino acid repeats that are polymorphic at positions 12 and 13, referred to as the repeat-variable di-residue (RVD) and determine EBE specificity; (3) two to three C-terminal nuclear localization signals (NLSs); and (4) an acidic transcription activation domain (AD) [17,22-25].

Among the 17 BLB resistance genes cloned, the functions of 14 *R* genes have been found to be related to TALEs except *Xa3/Xa26, Xa4* and *Xa21* [6,19]. *Xa1* and its alleles (*Xo1, Xa2, Xa14, Xa31(t)*, and *Xa45(t)*) encode NB-LRR type (NLR) proteins and confer resistance to *Xoo* by recognizing typical TALEs. However, the NLR resistance is suppressed independently on rice basal transcription factor TFIIAγ by iTALEs that are prevalent in Asian *Xoo* strains [8,9,16,26,27]. The recessive *R* gene *xa5* encodes a gamma subunit 5 of the basal transcription factor IIA (TFIIAγ5) and is a substitution variant of a single amino acid V39E. TFIIAγ5 and TFIIAγ1 directly interact with TALEs and are required for the survival of *Xoo* in rice [27-29]. Other recessive *R* genes, *xa13, xa25*, and *xa41*, encode SWEET family transmembrane proteins, which are basically sugar transporters, and the dominant alleles of these genes are *S* genes specifically induced by TALES for *Xoo* to establish infection [15,30-32]. The simultaneously disrupting TALE-EBEs of these *S* genes confers broad-spectrum resistance to rice against BLB [33,34]. *Xa7, Xa10, Xa23* and *Xa27* are known to be *E* genes triggered by TALEs AvrXa7/PthXo3, AvrXa10, AvrXa23 and AvrXa27, respectively [10,11,14,35,36]. The complexity of the interaction between TALEs and corresponding *R* genes is mystifying and has major implications for the continued deployment of stable resistance to *Xoo* in the field [6,37].

The *Xa23* gene has been widely implemented in rice breeding programs, both singly and in combination with other *R* genes [13,14,38,39]. Thus, it is important to monitor whether new *Xoo* isolates threaten *Xa23*-mediated resistance. In the present study, we collected 185 indigenous *Xoo* strains from various regions of China and recovered one isolate designated AH28 that was highly virulent on *Xa23*-containing rice line CBB23. To gain insight into the mechanism(s) by which AH28 overcomes *Xa23*, we sequenced its whole genome and assembled the full repertoire of TALEs. Further, isolation and ectopic expression of *tal7b* from AH28 revealed that one ortholog of *avrXa23* did not trigger resistance executed by *Xa23* in rice.

Materials and Methods

Plant material, bacterial strains and growth conditions

The susceptible rice varieties Kitaake, IR24, Nipponbare and near-isogenic resistant lines IRBB5 (harbouring *xa5*), IRBB7 (harbouring *Xa7*), IRBB10 (harbouring *Xa10*), IRBB13 (harbouring *xa13*) and CBB23 (harbouring *Xa23*) were grown in experimental fields and greenhouse located at Shanghai Jiao Tong University (Shanghai, China). The bacterial strains used in this study are listed in Table S3. *Escherichia coli* strains were grown in Luria-Bertani medium (LB) supplemented with appropriate antibiotics at 37 ℃. *Xoo* strains collected [33] were grown in nutrient broth (NB) or NB supplemented with 1.5% agar (NA) at 28 ℃ [40]. The rice lines and bacterial strains are stocked in Gongyou Chen's laboratory at Shanghai Jiao Tong University (Shanghai, China). Antibiotics were used at the following concentrations (μg/mL) when required: ampicillin (Ap), 100; kanamycin (Km) 25; and spectinomycin (Sp), 50.

Southern blot

For *tal* gene detection, *Xoo* genomic DNA was extracted, digested with *Bam*HI, separated in agarose gels, and transferred to membranes for blotting as previously reported [33,44]. The probe was made from a DNA fragment labeled with DIG containing the repetitive region of *pthXo1* (GenBank accession number: AY495676). Bacterial Genomic DNA Miniprep Kit was purchased from Axygen (USA). Restriction endonucleases and DNA molecular weight markers were provided by TaKaRa Bio (Japan). DIG-labeled Southern Blot kits were purchased from Roche (Switzerland) and Immobilon-Ny⁺ membranes were supplied by Millipore (USA).

AH28 genome sequencing, assembly, and annotation

AH28 Genomic DNA was extracted using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen, USA) and sequenced with the PromethION (Oxford Nanopore, UK) plus NovaSeq 6000 (Illumina, USA) by Shanghai OE Biotech Corporation (Shanghai, China) as described previously [41]. The sequencer was controlled with Min-KNOW version 2.2.12 software. A *de novo* genome assembly was performed with the software Flye version 2.6 [42] via default parameters. The Circos software [43] was used to generate the circular genome map of AH28 to show annotation information.

Genomes of AH28 and PXO99^A starting from the *gyrB* gene were aligned using progressive MAUVE with default parameters (http://darlinglab.org/mauve). The TALE coding genes (*tal* genes) were scanned using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and software AnnoTALE v1.2 [44]. The whole-genome sequence of AH28 was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and deposited in the NCBI BioProject Database (BioProject ID: PRJNA722377). The genome sequences of 20 *Xoo* strains were retrieved from the NCBI database and a phylogenetic tree was constructed by the Type Genome Server (TYGS) (https://tygs.dsmz.de).

Tal gene cloning and plasmid construction

tal7b of AH28 was isolated as described before [33,45]. Briefly, genomic DNA (2–3 μg) of AH28 was digested with *Bam*HI (New England Biolabs, USA) and separated in 1.3% agarose gels. Since the *Bam*HI fragment of *tal7b* is 4170 bp according to the genome sequence, DNA fragments larger than 4-kb were excised, ligated into *Bam*HI-digested pBluescript II KS(-), and transferred into *E. coli* DH5α. The resulting plasmid library was screened for TALE-containing clones by *in situ* colony blot hybridization using the 3.2-kb *Sph*I fragment of *avrXa23* (GenBank accession no. GU732172) as a probe. Hybridizing colonies were further evaluated by PCR analysis with *tal*-specific primers TALN18-F and TALN18-R (Table S4). Putative tal-containing clones were confirmed by restriction enzyme digestion and Sanger sequencing. *tal7b* and *avrXa23* was cloned into vector pZW in-frame with C-terminal FLAG-tag epitopes, resulting in pZW-tal7b and pZW-avrXa23 (Table S3). These plasmids were ligated into the broad-host-range vector pHM1 at the *Hind* III site, generating pHZW-tal7b and pHZW-avrXa23 (Table S3), which were then transferred into a *tal*-free strain PH (derived from PXO99^A) [16] and AH28, respectively, to obtain PH/tal7b, PH/avrXa23 and AH28/avrXa23 (Table S3).

Western blot

To detect the TALE proteins in *Xoo* strains, Western blotting was conducted as described previously [33]. The tested *Xoo* strains were cultured in NB to the logarithmic phase and harvested by centrifugation. Bacterial cells were washed twice, and adjusted to OD$_{600}$ = 2.0 with sterile distilled water. Proteins added loading buffer were boiled, separated on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane for immunoblotting with anti-FLAG (TransGen, China) as the primary antibody. Then the goat anti-rabbit IgG (TransGen, China) was used to detect the primary antibodies. The blotting was visualized with the EasySee Western Kit supplied by TransGen (China).

Disease assays

Xoo strains were cultured in NB supplemented with appropriate antibiotics at 28 °C for 20 h. Bacterial suspensions (OD$_{600}$ = 0.8) were used to inoculate two-month-old rice plants by the tip-cutting method [33]. Disease symptoms were recorded 14 days after inoculation (dpi), and lesion lengths (cm) were measured. For observation of water-soaking and the hypersensitive response (HR), strains (OD$_{600}$ = 0.6) were infiltrated into two-week-old rice seedlings with needleless syringes, and symptoms were recorded three days after infiltration. Five leaves were inoculated with each *Xoo* strain, and experiments were repeated three times.

Quantitative real-time PCR

Total RNA was isolated from inoculated plants 48 h post-inoculation using RNAiso Plus reagent (TaKaRa Bio, Japan). Trace amounts of genomic DNA were removed with RNase-free DNase I (TransGen, China) prior to the synthesis of cDNA. First-strand cDNA was diluted to a final volume of 20 μL, and SYBR green-labeled PCR fragments were amplified using *Xa23* gene-specific primers Xa23-F and Xa23-R (Table S4). in 7500 Real-Time PCR System (Applied Biosystems, USA). The rice ubiquitin gene was used as an internal control (primers Ub-F and Ub-R, Table S4). The comparative threshold ($2^{-\Delta\Delta Ct}$) method was used to calculate relative mRNA levels [46]. qRT-PCR experiments were performed in triplicate.

Electrophoretic mobility shift assay (EMSA)

avrXa23 and *tal7b* were cloned into the vector pET-30a with a His-tag to construct the plasmids pET30a-avrXa23 and pET30a-tal7b (Table S3). Induced by IPTG, the fusion protein His-AvrXa23 and His-Tal7b were expressed in *E. coli* BL21(DE3) cells containing pET30a-avrXa23 and pET30a-tal7b, respectively. Ni-NTA HisBind Resin (Novagen, USA) was used to purify the proteins according to the manufacturer's manual. Purified His-AvrXa23 and His-Tal7b were mixed with Cy5-labeled *Xa23* promoter fragments (probes synthesized by Shanghai DNA Bioscience Co. Ltd), respectively, and loaded on a 4.5% nondenaturing polyacrylamide gel for electrophoresis. Then an Amersham Typhoon RGB biomolecular imager (Cytiva, Sweden) was used to scan the fluorescence of the gel to detect the Cy5 fluorophore. Three independent experiments were taken and one similar result was displayed in this report.

Results

AH28 strain is compatible with Xa23-containing rice CBB23

A total of 185 *Xoo* strains were isolated from BLB-diseased rice leaves which were continuously collected in the past decades by this lab and most of them were listed in our previous report [33]. To detect the possibility that *Xoo* isolates counter *Xa23* resistance, all of them were inoculated to the near-isogenic rice lines (NILs) with different *R* genes by the tip-cutting method. Interestingly, the AH28 strain, which was originally isolated from Anhui province, is incompatible with rice IRBB5 (*xa5*) and IRBB7 (*Xa7*), but compatible with rice IRBB10 (*Xa10*), IRBB13 (*xa13*) and CBB23 (*Xa23*) (Table 1). The mean lesion length in CBB23 caused by AH28 was 12.42 cm, longer than that (0.23 cm) by PXO99^A (Table 1). These results indicate that AH28 possesses unknown virulence factor(s), which is(are) different from PXO99^A and overcome(s) *Xa23*-mediated resistance in rice. Given that *Xa23* confers the broadest spectrum resistance to BLB [13,14], it is possible that AH28 is an emerging minority naturally co-evolved to challenge such resistance pressure in rice fields.

Table 1
Inoculation results of *Xoo* strain AH28 and PXO99[A] with NILs.

NILs[a]	Strains	Lesion length (cm)	Phenotypes[b]	TALEs matched
IRBB5(*xa5*)	AH28	0.58	R	PthXo7 absent
	PXO99[A]	6.32	S	PthXo7
IRBB7(*Xa7*)	AH28	0.33	R	PthXo3
	PXO99[A]	12.23	S	AvrXa7 and PthXo3 absent
IRBB10(*Xa10*)	AH28	13.38	S	AvrXa10 absent
	PXO99[A]	13.01	S	
IRBB13(*xa13*)	AH28	13.02	S	PthXo2 and PthXo3
	PXO99[A]	1.87	R	PthXo1
CBB23(*Xa23*)	AH28	20.2	S	AvrXa23-like TALE
	PXO99[A]	1.43	R	AvrXa23
Nipponbare	AH28	13.42	S	
	PXO99[A]	13.45	S	
IR24	AH28	13.75	S	
	PXO99[A]	12.17	S	

[a] NILs, near-isogenic lines rice.
[b] S, susceptibility with lesion length > 2.5 cm; R, resistance with lesion length ≤ 2.5 cm.

Whole-genome sequence analysis of AH28

To gain insights into the reason why *Xa23* resistance is overcome, we completed the whole genome sequence of AH28 using long-read Nanopore sequencing combined with Illumina genome sequencing. The genome was assembled into two contigs, corresponding to a circular chromosome of 4,923,022 bp (accession number CP074076) and a plasmid pAH28 of 42,144 bp (accession number CP074077), respectively (Fig. 1). The GC contents of the chromosome and plasmid were 63.71% and 61.95%, respectively (Table S1). The AH28 genome contains 4,816 predicted genes, of which 3,407 (70.7%) genes encode proteins categorized into clusters of orthologous groups (COG) category (Table S1). A comprehensive frame of the AH28 genome is presented in Fig. 1,

including the predicted 17 *tal* genes (red color) and the COG function classification of genes.

To further investigate the population structure of *Xoo* strains, we performed the phylogenetic tree analysis using genome sequences of AH28 and other 19 fully sequenced *Xoo* strains, including 11 Chinese strains (Fig. 2, Table S2). The 11 Chinese strains could be divided into three clades (Fig. 2): Clade I contained two strains from Liaoning province of China (LN18 and LN4) and nine strains in Clade II were from Jilin, Jiangsu, Hunan, Sichuan, Taiwan and Anhui provinces, including the newly sequenced AH28. A strain from Yunnan (YN24) was in Clade III, which also contained an Indian strain (IX-280) and a Philippine strain PXO99[A]. These results implied that the geographical distribution of *Xoo* strains may be reflected in the genome features. Indeed, the
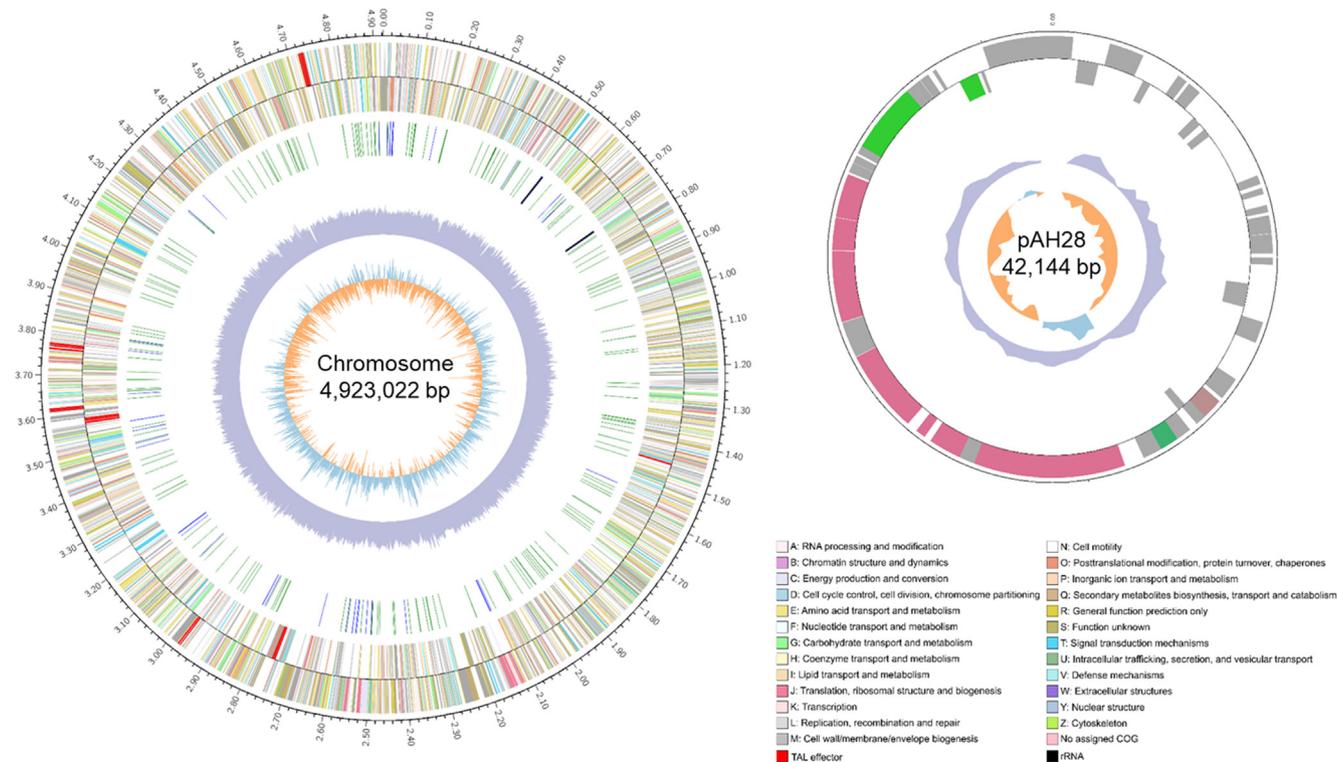

Fig. 1. Circular genome map of AH28 strain. The outermost ring is genome size, with 0.1 Mb intervals. The second and the third circle are CDS on the sense and antisense strands. Different colors indicate CDS according to COG functional classification. The fourth circle represents noncoding RNA. The fifth circle shows the GC content. Central circle is the GC skew value.
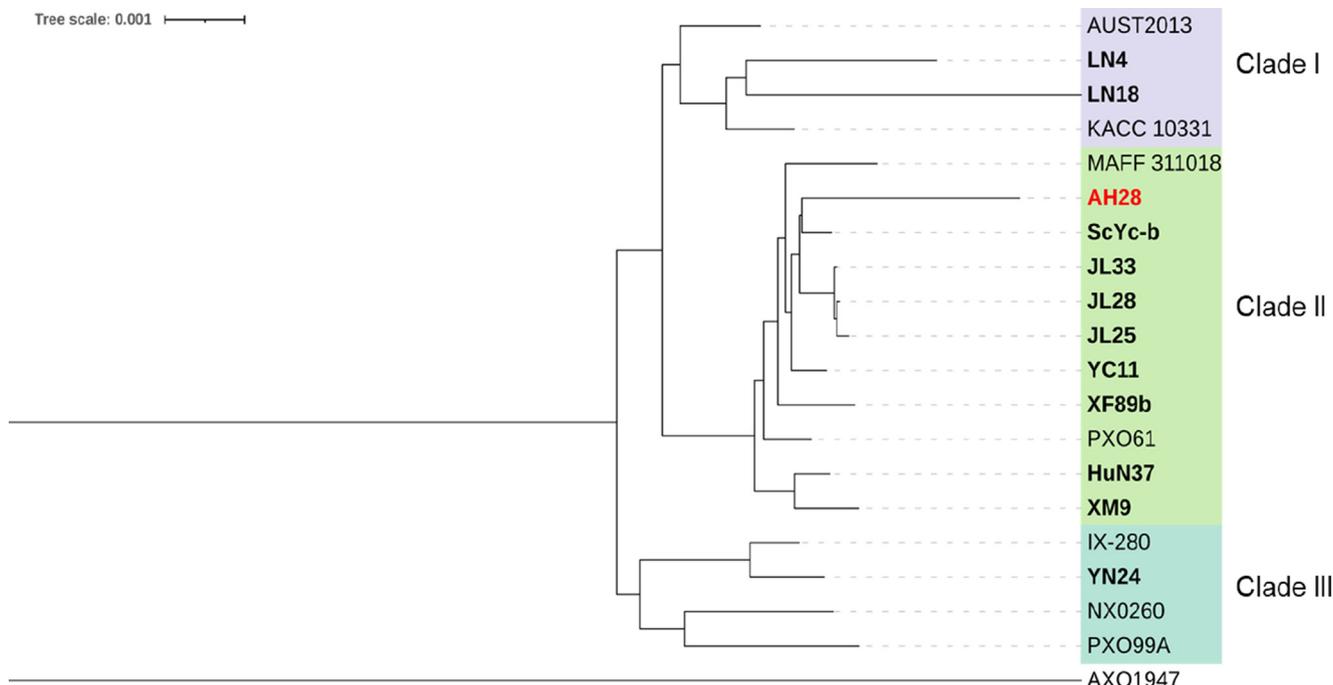
Fig. 2. Phylogenetic tree of 20 fully sequenced *Xoo* strains. 19 fully sequenced Asian *Xoo* strains were used to create a phylogenetic tree based on genomic sequences. The African *Xoo* strain AXO1947 was used as an outgroup.

MAUVE alignments of AH28 and PXO99^A genomes showed a high-level genomic rearrangements and inversions (Fig. 3A), adapting to different rice cultivars planted geographically.

TALEs encoded by the AH28 genome

Genome sequence analysis showed that there were seven *tal* gene loci in AH28 encoding 17 TALEs and eight loci encoding 19

TALEs in PXO99^A (Fig. 3B). The TALE repertoire of AH28 consisted 15 typical TALEs and 2 iTALEs (Fig. 3B, 4, S1). The iTALEs are TALE variants with shortened and truncated N- and C-termini that function as the suppressors of the resistance mediated by *Xa1/Xo1* and their alleles [8,9,16,26]. The predicted RVD numbers in each of AH28 TALome varies from 13 to 29 (Fig. 4). Seven TALEs in AH28 are identical to those in the genomes of the *Xoo* strains available in the NCBI database, and ten TALEs in AH28 displayed one to nine
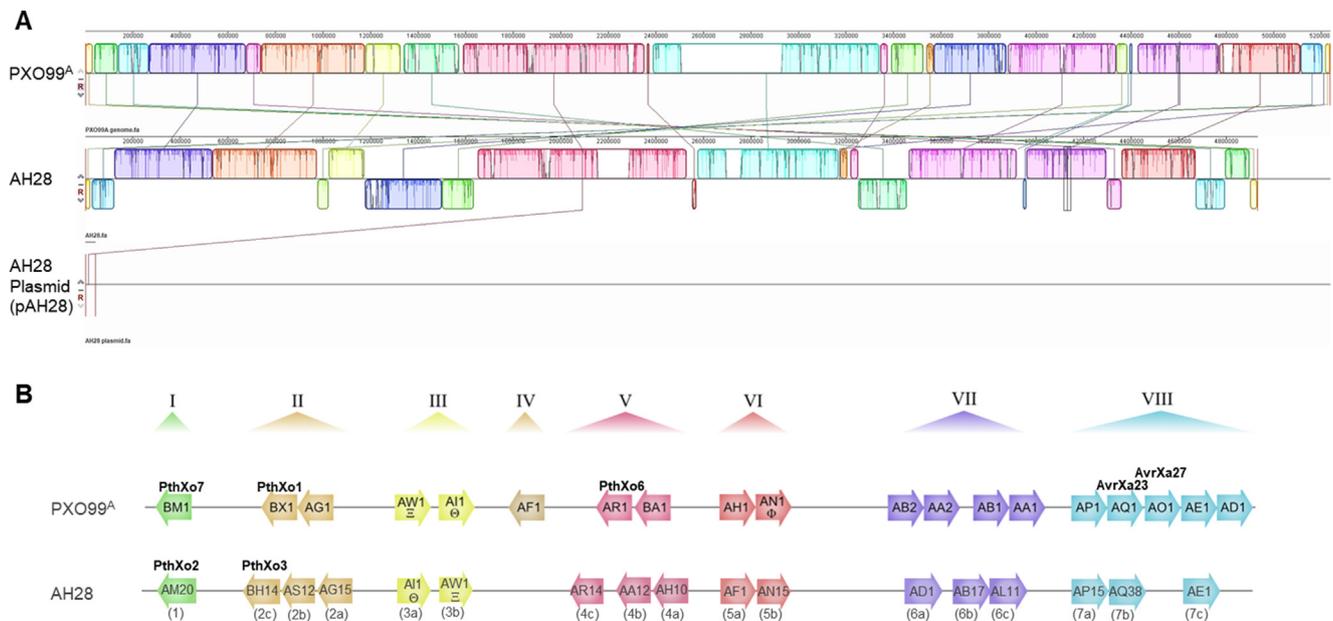

Fig. 3. Comparison of whole genomes and *tal* genes of AH28 and PXO99^A. **A**, Progressive Mauve alignment of the AH28 and PXO99^A genomes. AH28 genome comprises a single circular chromosome and a single circular plasmid, shown linearized. Blocks of the same color indicate similar genomic regions. **B**, TALome overview of PXO99^A and AH28. *tal* genes are represented by arrows indicating their relative orientation in the genome. All TALEs are assigned into classes by AnnoTALE and named accordingly, while the alternative name of prominent members of the classes is indicated in bold. iTALEs are labeled with xi(Ξ) for iTALE type A or theta ((Θ)) for iTALE type B. Previously established TALE clusters are specified at the top and cluster affiliation of individual *tal* genes is shown by color.

RVDs different to those possessed by PXO99[A] (Fig. 4). These results suggest that the TALome of AH28 is acquired for the co-evolution of the pathogen virulence with its host rice in a particular rice-growing region.

The TALome overview of AH28 showed seven clustered *tal* genes without the cluster IV, which were present in PXO99[A] (Fig. 3B). The cluster I in AH28 contained one *tal* gene (*tal1*) encoding PthXo2, whereas PthXo7 correspondingly in PXO99[A] (Fig. 3B). These explained that the incompatibility of AH28 with IRBB5 (*xa5*) rice was due to the absence of PthXo7 in the AH28 genome (Table 1). The cluster II had three *tal* genes in AH28, including *tal2c* for PthXo3 (Fig. 3B, 4), which activated the expression of *Xa7* for ETI and *OsSWEET14* for ETS in rice [10,11,15]. This likely explains that the strain AH28 was incompatible with *Xa7*-containing rice IRBB7, while PXO99[A] was compatible (Table 1). The two major virulence TALEs, PthXo2 and PthXo3 enabled the strain AH28 to be susceptible in rice IRBB13 (*xa13*), but are absent in PXO99[A], which was incompatible with IRBB13 (Table 1). Interestingly, AvrXa10, matched by *Xa10* [36], is absent both in AH28 and PXO99[A], explaining the reason that these two strains are compatible with IRBB10 (*Xa10*) (Table 1, Fig. 4).

The compatibility of AH28 with rice CBB23 (*Xa23*) (Table 1) and no similar *Xa23*-hybridized band showed in AH28 (Fig. S1) compromises us to assume that the *Xa23*-matched avirulence gene *avrXa23* may be absent or mutated in AH28. With the help of the AH28 genome sequence, it is surprising to see an ortholog of *avrXa23* gene, *tal7b*, is present in the strain AH28 (Fig. 3B, 4, S1). Then, *tal7b* was directly cloned by cutting the putative *tal*-hybridized band (Fig. S1) through the screening by *in situ* colony hybridization. The sequencing results, including the genome

sequence, showed that *tal7b* is 4347 bp in size with 97.2% identity at the nucleotide levels to *avrXa23* of PXO99[A] and encodes an AvrXa23-like TALE (designated Tal7b or AvrXa23C), totally 1448 amino acid residues with 26 RVD repeats (Fig. 4). Compared with AvrXa23, Tal7b has the 4th repeat missed, and the 5th and 8th RVDs changed from NG and NS to NS and S*, respectively (Table 2). The information above implies that Tal7b may be a mutation of AvrXa23.

Tal7b, an AvrXa23-like TALE, did not trigger resistance mediated by Xa23 in rice

To verify the hypothesis in the previous section, the isolated *tal7b* was subcloned as FLAG-tagged derivatives into pZW, resulting in pZW-tal7b (Table S3), and this construct was then inserted into the *Hind*III site of pHM1, resulting in the cosmid pHZW-ta7b (Table S3). The construct was then transferred into *Xoo* PH, a *tal*-free derivative of PXO99[A] [16], generating a strain PH/tal7b (Table S3). The cosmid pHZW-avrXa23 was constructed in the similar strategy mentioned above and then transferred into PH and AH28 strains, resulting in PH/avrXa23, and AH28/avrXa23, respectively (Table S3). Western blot analysis showed that both Tal7b and AvrXa23, about 150 kDa, were detectable in corresponding *Xoo* strains (Fig. 5A). *Xoo* strains containing pHZW-avrXa23, pHZW-ta7b or empty vector (EV) were infiltrated into young CBB23 seedling leaves using needleless syringes. As expected, Tal7b, the mutated version of AvrXa23, did not make the PH strain trigger HR on CBB23, while AvrXa23 enabled AH28 from compatibility (water-soaked symptoms) to incompatibility (HR phenotype) with CBB23 when AH28 and PH harbored the *avrXa23* gene *in trans*

TALE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	PXO99[A] Ortholog
Tal1 (PthXo2)	NI	HG	NI	NN	NN	NI	NN	HD	NI	HD	NS	NS	NS	HD	NN	HD	NG	HD	HD	HD	NG	NG								/
Tal2c (PthXo3)	NI	HG	NI	HG	NI	NI	NI	HD	NN	HD	HD	HD	NG	HD	N*	NI	HD	HD	NN	NS	NI	NN	NN	NG	NN	HD	N*	NS	N*	/
Tal2b	NI	HG	NI	NI	HG	HD	NN	HD	HD	HD	NI	NI	NN	NI	HD	HD	HD	HG	NN	NN	HD	NS	NN	HD	NG	NS	N*			/
Tal2a	NI	NG	NN	NG	NK	NG	NI	NN	NI	NN	NI	NN	NS	NG	NS	NN	NI	N*	NS	NG										Tal2a
Tal3a†	NS	HD	NG	NG	NG	NG	HD	HD	HD	HD	NN	HD	HD	HD	HD	H*														Tal3b
Tal3b†	NS	HD	NG	NG	NG	NG	HD	HD	HD	HD	NN	HD	NG	HD	NI	HD	NN	N*												Tal3a
Tal4c	NI	H*	NI	NN	NN	NN	NN	NN	HD	NI	NS	HG	HD	NI	N*	NS	NI	NI	HD	HD	N*	NS	N*							PthXo6
Tal4b	NI	HG	NI	NG	HG	HD	NS	NG	HD	NN	NG	HG	NG	HD	HG	HD	HD	NI	NN	NG										Tal8b
Tal4a	NI	N*	NI	NS	NN	NG	NN	NS	N*	NS	NN	NS	N*	HD	HG	HD	NI	HD	HD	NG										Tal6a
Tal5a	NI	NN	NN	NI	NI	NI	HD	NS	HG	NN	NN	NN	NI	NI	NG	HD														Tal4
Tal5b	NI	N*	NI	HG	NI	NI	NS	HD	NN	HD	NS	NG	SS	NN	NI	NI	NN	NI	NN	NI	NG									Tal6b
Tal6a	NN	HD	NS	NG	HD	NN	N*	NI	HD	NS	HD	NN	HD	NN	HD	NN	NN	NN	NN	NN	NN	HD	NG							Tal9e
Tal6b	NI	HG	NI	NI	NI	NN	HD	NS	NN	NS	NN	HD	NN	NI	HD	NN	NI	NG	HD	NG										Tal7a/8a
Tal6c	NI	NS	HD	NG	NS	NN	HD	N*	NN	NN	NI	NG	HD	NG	HD	HD	HD	NG												/
Tal7a	HD	HD	HD	NG	N*	NG	HD	S*	HG	NI	NI	NN	HD	NN	ND	HD	NI	HD	HG	NG										PthXo8
Tal7b	HD	HD	NN	NS	NG	HD	S*	HG	HD	NG	N*	HD	HD	HD	N*	NN	NI	NN	HD	HI	ND	HD	HG	NN	HG	N*				AvrXa23
Tal7c	NI	NN	NI	HG	HG	NV	HG	HD	HG	HD	HD	HD	NG																	Tal9d

Fig. 4. RVD sequences of AH28 TALEs. RVDs in red color are different in PXO99[A] orthologs. An asterisk indicates that the second amino acid in the RVD is absent, resulting in a 33 aa repeat. A dagger indicates an iTALE.

Table 2
Multiple alignment of the RVD sequences of AvrXa23-like TALEs.

TALEs	RVDs[a]	Strains[b]
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28	
AvrXa23 (9.52%)	HD HD NN NN NG NG HD NS HG HD NG N* HD HD HD N* NN NI NN HD HI ND HD HG – NN HG N*	PXO99[A], PXO79, ICMP3125, IXO1088
AvrXa23A (35.71%)	HD HD NN NN NS NG HD S* HG HD NG N* HD HD HD N* NN NI NN HD HI ND HD HG – NN HG N*	MAFF 311018(T7174), PXO86, PXO83, PXO211, PXO236, PXO563, K3, K3a, JL25, JL28, YC11, JP01, ScYc-b, XM9, PXO145
AvrXa23B (2.38%)	HD HD NN NG NG HD NS HG HD NG N* HD HD HD N* NN – NN HD HI ND HD HG HG NN HG N*	NXO260
AvrXa23C (2.38%)	HD HD NN – NS NG HD S* HG HD NG N* HD HD HD N* NN NI NN HD HI ND HD HG – NN HG N*	AH28
AvrXa23D (45.24%)	HD HD NN NN NI NG HD S* HG HD NG N* NG HD HD N* NI NI NN HD HI ND HD NG – NN HG N*	LN18, LN4, PXO61, PXO71, PXO524, PXO602, IXO704, IX-280, SK2-3, BXO1, PXO404, PXO421, PXO513, JW11089, XF89b, K1, K2, KXO85, AUST2013
AvrXa23E (2.38%)	HD HD NN NN NI NG HD S* HG HD NG N* – HD HD N* NI NI NN HD HI ND HD NG – NN HG N*	PXO282
AvrXa23F (2.38%)	HD HD NN NN NI NG HD S* HG HD NG N* NG HD N* N* – NI NN HD HI ND HD NG – NN HG N*	PXO142

[a] RVDs that are different from those of the reference (AvrXa23) at the same positions are colored in red font. S* are rare RVDs in TALomes of *Xanthomonas* species.
[b] Strains in blue means incompatible with *Xa23* rice tested by the report (Wang et al., 2014).

(Fig. 5B). The incompatibility was similar to that caused by PXO99[A], which contains the *avrXa23* gene in the genome (Fig. 3B). The results above suggest that the AvrXa23-like TALE, Tal7b, is a mutant of AvrXa23, which does not act as an avirulence factor to trigger *Xa23* resistance in rice. On the other hand, Tal7b did not contribute increased-virulence to the PH strain when BLB-susceptible rice Kitaake was used for virulence detection by tip-cutting method (Fig. 5C), indicating that Tal7b is not a major virulence TALE.

To ascertain whether Tal7b could activate *Xa23* expression in CBB23 rice, a qRT-PCR method was used. The expression of *Xa23* was not induced in CBB23 leaves, which were infiltrated with bacterial cells of AH28, PH/tal7b and PH/EV (negative control), respectively, but significantly increased when PH/avrXa23 or AH8/avrXa23 was inoculated (Fig. 5D). Collectively, these results imply that Tal7b is the mutant of AvrXa23 and loses the avirulence to match *Xa23*.

Given that the activation of *Xa23* by AvrXa23 is dependent on the EBE (Fig. 5E) present in the *Xa23* promoter [14], we subsequently investigated whether or not Tal7b binds to the EBE of *Xa23*. The EMSA results showed that Tal7b did not bind the EBE, while AvrXa23 did (Fig. 5F).

Prevalence of AvrXa23-like TALEs in Xoo strains

The *avrXa23* gene or its functional equivalent is previously identified only in strains PXO99[A] and MAFF 311,018 (T7174), and is speculated to be ubiquitous among *Xoo* strains [13]. To validate this, we investigated the distribution of *avrXa23* gene or its homologous sequences by searching the complete genome sequences of 83 *Xoo* strains loaded in the NCBI database by BLAST. These strains represent diverse geographic areas, consisting of 47, 34, 1 and 1 strains from Asia, Africa, South America and Oceania, respectively. The results showed that a total of 42 *Xoo* strains contain AvrXa23-like TALE, while 41 strains, including all the African and South American strains and six Asian strains, do not (Table S2). AvrXa23-like TALEs in these 42 *Xoo* strains were classified into seven versions according to their RVD sequences (Table 2): (1) AvrXa23 presents in four strains (PXO99[A], PXO79, ICMP3125 and IXO1088); (2) AvrXa23A in fifteen strains, which has the 5th (NS) and 8th (S*) RVDs changed, compared to AvrXa23; (3) AvrXa23B in one strain NX0260, the 18th RVDs (NI) missed and the 25th (HG) added; (4) AvrXa23C in our strain AH28, the 4th (NN) missed and the 5th (NS) and the 8th (S*) changed; (5) AvrXa23D present in

nineteen strains, the 5th, 8th, 13th, 17th, and 24th different from those of AvrXa23; (6) AvrXa23E in a strain PXO282, the 13th missed; and (7) AvrXa23F in a strain PXO142, the 15th changed to N* and the 17th missed in the comparison with AvrXa23D (Table 2). These RVD differences showed diversities of AvrXa23-like TALEs in *Xoo* population, suggesting that the *avrXa23* locus may undergo the resistance pressure by the cognate *R* gene. Moreover, AvrXa23D seems like the major version of AvrXa23-like TALEs displayed from the available genome sequences of *Xoo* strains so far (Table 2).

Limited by the plant quarantine policies among countries, four *Xoo* strains, PXO99[A], PXO86, AH28 and LN18, which contain AvrXa23, AvrXa23A, AvrXa23C, and AvrXa23D, respectively, were chosen as the representatives of the *Xoo* collection to confirm the gene-for-gene manner between the AvrXa23-like TALEs and the *Xa23* gene. Indeed, the AvrXa23C-harbored strain AH28 was compatible with *Xa23*-containing rice CBB23, while the other three strains were incompatible (Fig. S2), implying that AH28 is an emerging race to overcome *Xa23* resistance in rice.

Discussion

Xa23, a TALE-dependent *E* gene, confers the broadest resistance against *Xoo* strains without any exception before this report [13,14,47]. Here, we uncovered an *Xa23*-breaking *Xoo* isolate, AH28 from a rice-growing field in Anhui province of China (Table 1). The genome sequencing (Figs. 1, 3) and TALE analysis (Fig. 4, S1) of this isolate led to the finding that the Tal7b, with the 4th RVDs (NN) missed and the 5th (NS) and the 8th (S*) changed in comparison with those of AvrXa23, is an AvrXa23-like TALE that makes the pathogen unable to trigger *Xa23*-mediated ETI (Fig. 5, S2). This is consistent with the binding affinity of AvrXa23, but not Tal7b, to the EBE of *Xa23* promoter (Fig. 5F).

The concept accepted is that the induction of *S* or *E* genes via the EBEs bound by the presence of major virulence factors (PthXo) or matching avirulence factors (TALEs) confers susceptibility or resistance to rice. The completed genomes show that the strain AH28 has PthXo2 and PthXo3, but not PthXo1 and PthXo7, which are present in PXO99[A] (Fig. 3B), explaining the incompatibility of AH28 with *Xa7*-containing rice IRBB7 and *xa5*-containing rice IRBB5 vs the compatibility of PXO99[A] with IRBB7 and IRBB5, respectively (Table 1), since *OsSWEET11* is targeted by PthXo1 [20], *OsSWEET13* by PthXo2 [31], *Xa7* by PthXo3 or AvrXa7 [10,11], and *OsTFIIAγ1* by PthXo7 [48]. The absence of AvrXa10 both in AH28 and PXO99[A]
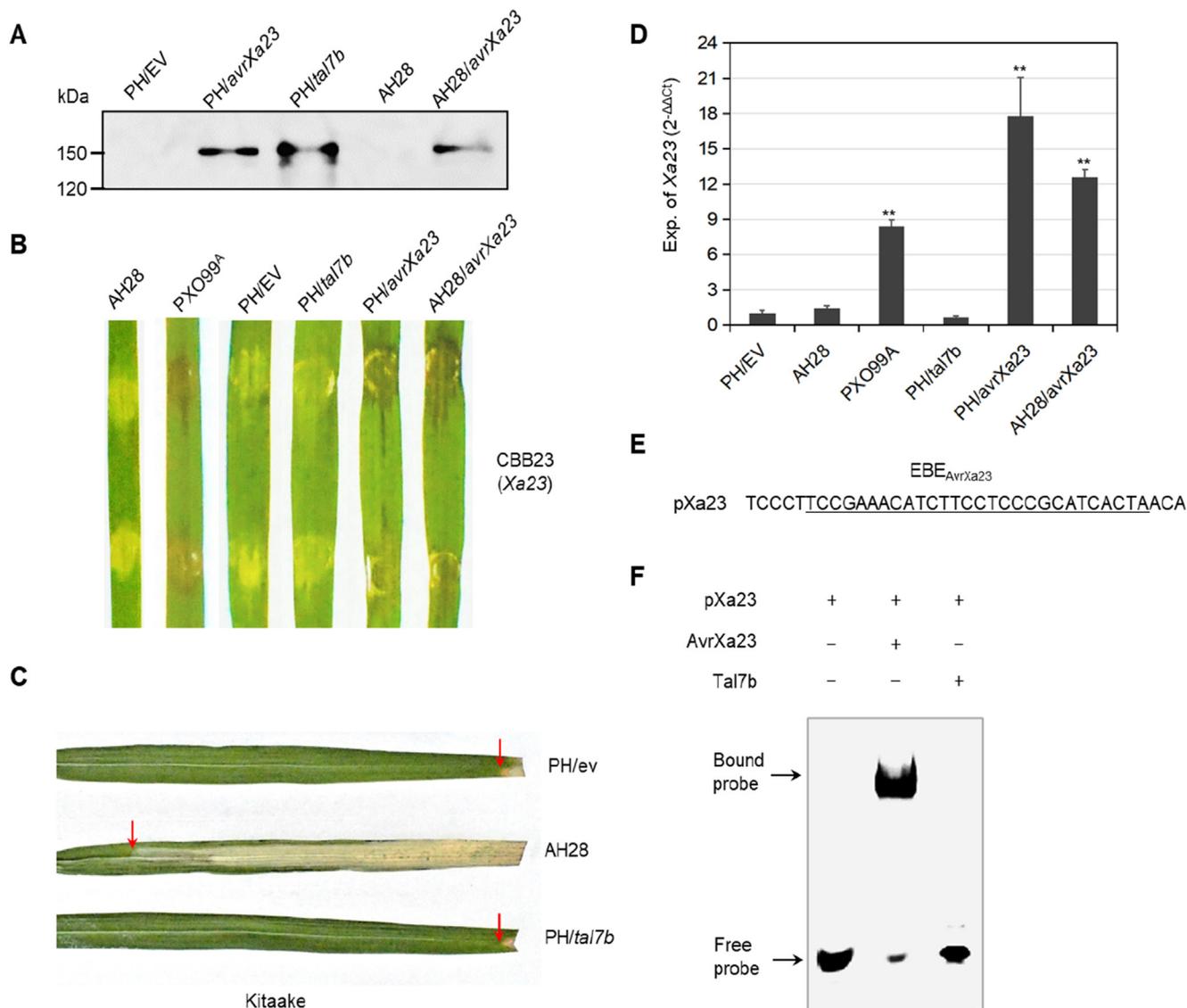
Fig. 5. *tal7b* of AH28 strain could not activate *Xa23*-mediated resistance in CBB23. **A**, *tal7b* and *avrXa23* expression in *Xoo* PH and AH28 containing the FLAG-tagged, broad-host range construct pHZW-tal7b or pHZW-avrXa23. Bacterial extracts were separated by SDS-PAGE, blotted, and probed with anti-FLAG antisera. **B**, Reaction of CBB23 rice leaves to *Xoo* AH28, PX099^A and *Xoo* transformants containing pHZW-tal7b or pHZW-avrXa23. Bacterial suspensions were infiltrated into rice leaves using needleless syringes and photographed three days post-inoculation. **C**, Phenotypes of disease reactions in Kitaake after inoculation with *Xoo* AH28 and PH derivatives containing the empty vector (EV) or *tal7b*. **D**, Expression of *Xa23* in CBB23 rice leaves inoculated with *Xoo* strains. The expression of *Xa23* was measured in rice leaves infiltrated with *Xoo* PH/EV, AH28, PXO99^A, PH/tal7b, PH/avrXa23 and AH28/avrXa23. RNA was extracted from leaves 48 hpi and used for qRT-PCR with *Xa23*-specific primers. The expression level of the rice ubiquitin gene was used as an internal control. Columns marked with asterisks (**) represent statistically significant results (P ≤ 0.01) as deduced from a paired, two-tailed, student's *t*-test. **E**, Nucleotide sequence of *Xa23* promoter fragment (probe). The EBE$_{AvrXa23}$ is underlined. **F**, EMSA with the His-Tal7b and His-AvrXa23 fusion proteins and a Cy5-labeled *Xa23* promoter fragment (probe). Positions of the bound and free probe are indicated at the left.

(Fig. 3B) makes the strains compatible with *Xa10*-containing rice IRBB10 (Table 1). It also could be predicted that AH28 is compatible with *Xa27*-containing rice (though we did not test this in the report), since it does not have the TALE AvrXa27 (Fig. 3B). Several *Xa27*-breaking *Xoo* strains have been reported before [13]. Thus, there is no exception that the resistance mediated by these four *E* genes could not be overcome by the pathogen presently, suggesting that durable broad-spectrum resistance for BLB mediated by an *E* gene is relative to an emerging race, whether dominant or minority.

The arms-race between the pathogen and the host normally occurs in a gene-for-gene manner. PthXo2 secreted by *Xoo* as a major virulence TALE targets the *S* gene *OsSWEET13* to induce ETS [31]. Five PthXo2 variants and ten haplotypes of *OsSWEET13* have been identified in *Xoo* strains and rice varieties, respectively, which is the result of arms-race between *Xoo* and rice [33]. For ETI

triggered by an *avr* gene and the corresponding *R* gene, pathogen may mutate or just discard the *avr* gene under high selection pressure generated by the *R* gene [15]. In this study, we found that AH28 loses *avrXa10* but contain a defective copy of *avrXa23*, *tal7b* (Table 1, Fig. 4, S1). Seven versions of AvrXa23-like TALEs were also identified in different *Xoo* strains (Table 2). These results demonstrate that diversified strategies are used by *Xoo* population to evade recognition by *E* genes in rice.

Combining the results of this study and previous reports [10,11,14,20,31], we propose that *avrXa23*-containing *Xoo* strains utilize major TALEs to target the corresponding *S* genes and induce ETS, which can be suppressed by *Xa23*-mediated ETI triggered by AvrXa23, leading to host resistance to these strains; The *Xa23*-breaking isolate AH28 modifies the RVDs of AvrXa23 to avoid binding to the EBE and activating the expression of *Xa23* in rice, which would impede the inhibitory effect of ETI on ETS induced by major
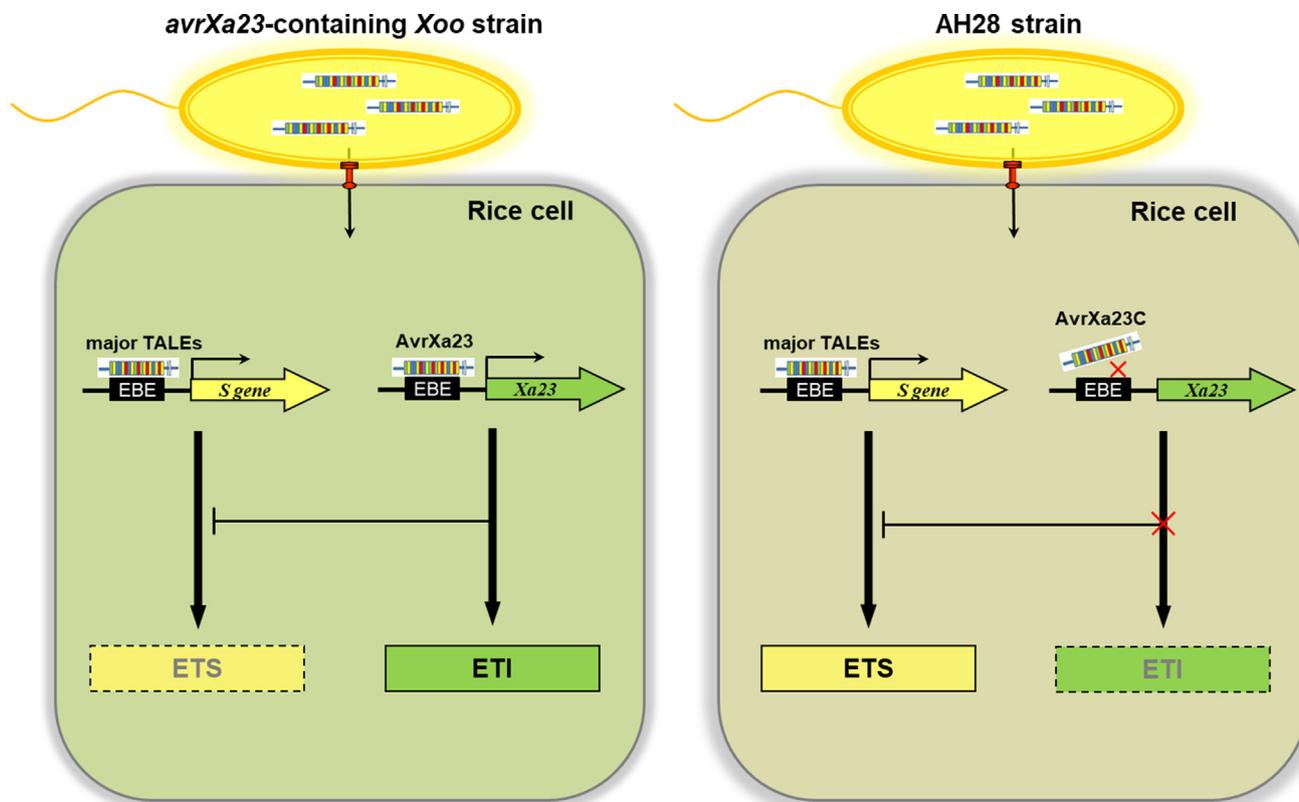
Fig. 6. Proposed model for the strategy used by *Xoo* to overcome *Xa23*-mediated resistance. The ETI conferred by *Xa23* is dependent on the binding of the cognate AvrXa23 to the EBE of *Xa23*. AH28 strain modify the RVDs of AvrXa23 to avoid binding to the EBE and activating the expression of *Xa23* in rice, which would impede the inhibitory effect of ETI on ETS induced by major TALEs (e.g. PthXo2 and PthXo3), resulting in host susceptibility to the strain.

TALEs (PthXo2 and PthXo3), resulting in host susceptibility to the strain (Fig. 6). However, the mechanism of *Xa23*-mediated ETI is still unclear and further research is needed.

None of the seven AvrXa23-like TALEs was present in the genomes of six Asian *Xoo* strains including three Chinese strains (Table S2). One of the strains, HuN37 originally isolated from Hunan province of China, was inoculated to the *Xa23*-containing rice line CBB23 (Table S2, Fig. S2). Surprisingly, this strain is incompatible in CBB23 (Fig. S2), which may be explained by the inaccurate assembly of this genome or other effector(s) in this strain activating host resistance. This requires further experiments to determine.

The broad-spectrum resistance mediated by *Xa23* to *Xoo* has led to its inclusion in rice breeding programs, and several *Xa23*-containing elite inbred and hybrid rice varieties have been released to growers [13,14,38,39]. However, our discovery shows that there is a potential, emerging *Xoo* population that can overcome *Xa23*-mediated BLB resistance. Thus it is important to continually monitor the *Xoo* population in the world, particularly in China by genome sequencing and TALome analysis. Meanwhile, it is necessary to avoid planting rice varieties with a single *R* gene (like *Xa23*) in a large area. The diversified implementation of multiple resistance strategies, including those that exploit the EBE site of *E* genes and *S* genes, are promising approaches to stay abreast of the ongoing 'arms race' between *Xoo* and cultivated rice [33,34,37,47,49-51].

Conclusion

In conclusion, *Xoo* may survive by changing few RVDs of an avirulence TALE to avoid being trapped by the EBE of a matched executor *R* gene in rice. This may impede the inhibitory effect of ETI on ETS to keep host susceptibility (Fig. 6).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.01.007.

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