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PthXo2B Orthologue Tal7 of *Xanthomonas oryzae* pv. *oryzae* Strain IX-221 Acts as a Major Virulence Factor in *Indica* Rice Without Activating a Clade III *SWEET* Gene

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ABSTRACT

In rice bacterial blight, *Xanthomonas oryzae* pv. *oryzae* deploys *t*ranscription *a*ctivator-*l*ike *e*ffectors (TALEs) that upregulate host susceptibility genes. Thirty-four amino acid repeats in TALEs each specify a base in the DNA target, via a repeat-variable diresidue (RVD; positions 12 and 13). Some aberrant-length repeats can disengage to accommodate single base deletions. Clade III *SWEET* genes *SWEET11*, *-13* and *-14* are major susceptibility targets of different TALEs. *xa13* is a *SWEET11* allele lacking the TALE binding site and thus confers resistance to some strains. It has been deployed widely in India. We report that an *xa13*-breaking Indian isolate, IX-221, harbours one *SWEET14*- and two *SWEET13*-activating TALEs, with one or two disengageable repeats. One, Tal7, orthologous to PthXo2B of Philippines strain PXO61 but with minor, non-RVD sequence differences, like PthXo2B upregulates *SWEET13* in a *japonica* variety and no clade III *SWEET* in an *indica*, yet unlike PthXo2B renders both varieties susceptible. A designer TALE with distinct, minor differences also failed to render the *indica* susceptible. The results suggest that Tal7 activates an alternative susceptibility gene and that non-RVD polymorphism can affect TALE targeting. Moreover, IX-221 provides evidence that the deployment of *xa13* in India resulted in strains super-equipped with TALEs that break it.

Xanthomonas oryzae pv. oryzae (Xoo) causes rice bacterial blight. Xoo injects transcription activator-like effectors (TALEs) that target effector-specific binding elements (EBEs) in rice gene promoters and increase transcription (Bogdanove et al. 2010). Such genes that contribute to disease are 'susceptibility' (S) genes. A central repeat region (CRR) in TALEs governs target recognition. It consists of tandem, 33–35 amino acid (aa) repeats, differing at positions 12 and 13, together called the repeat-variable diresidue (RVD). Following a partially degenerate code, each RVD directly interacts with a single nucleotide. The number and composition of RVDs thus predict the EBE sequence (Moscou and Bogdanove 2009; Boch et al. 2009). Besides the standard-length repeats (Wilkins et al. 2015; Oliva et al. 2019; Richter et al. 2014), variants of 28, 30, 36, 39, 40 and 42 aa have been reported. Some of these so-called aberrant repeats can function as a standard repeat or disengage to accommodate a 1 base deletion and maintain register downstream (Becker et al. 2022).

Members of clade III of the Sugars Will Eventually be Exported Transporters (SWEET) gene family are major rice bacterial

blight S genes (Streubel et al. 2013). Their cognate TALEs are called major TALEs. The first *SWEET*–TALE interaction identified was *SWEET11* with PthXo1, a TALE found in the Philippines strain PXO99 (Chu et al. 2006; Yang et al. 2006). *SWEET13* is activated by PthXo2, identified first in Xoo strains from Japan (Yang and White 2004; Ochiai et al. 2005; Zhou et al. 2015). Finally, *SWEET14* is activated by TALEs AvrXa7, PthXo3, TalC and Tal5, from geographically diverse strains (Antony et al. 2010; Yu et al. 2011; Streubel et al. 2013). Tables S1, S7 compiles these TALEs and targets, as well as others described below.

SWEET allelic variation at the EBE that prevents activation by the cognate TALE, and *SWEET* allele-specific variants of some major TALEs have been described. For example, the recessive bacterial blight resistance gene *xa13* was revealed to be any of several alleles of *SWEET11* with promoter mutations that disrupt the PthXo1 EBE, providing resistance (loss of susceptibility) to strains that depend on PthXo1 (Chu et al. 2006). Also, PthXo2 activates *SWEET13* only in *indica* rice; the *japonica* allele has a single nucleotide difference at the EBE that prevents PthXo2 binding and renders the plant resistant, and this allele was determined to be the *xa25* resistance gene (Zhou et al. 2015). A variant of PthXo2 named PthXo2B, found in a few Xoo strains, has aberrant (36 aa) 9th and 12th repeats and activates the *japonica* but not the *indica* allele of *SWEET13*, rendering *japonica* genotypes susceptible (and overcoming *xa25*) (Oliva et al. 2019). In addition to such adaptations, some strains harbour more than one major TALE, enabling them to overcome an allelic variant of one *SWEET* gene by simultaneously targeting another *SWEET* gene (Oliva et al. 2019). These observations demonstrate that *SWEET*–TALE interactions are a key molecular interface for adaptation and counteradaptation of rice and Xoo.

xa13 has been used widely in rice breeding since the development of molecular markers for it in the mid-1990s (Zhang et al. 1996; Fiyaz et al. 2022). It has been largely effective, especially when stacked with other resistance genes (Fiyaz et al. 2022). However, in India, where it has been in use since at least 2001 (Singh et al. 2001), *xa13* resistance has broken down repeatedly (Mishra et al. 2013; Yugander et al. 2017; Midha et al. 2017; Mondal et al. 2014). Yugander et al. (2017), in a study of 400 isolates from across the country, found 134 against which *xa13* is ineffective. These were distributed across 10 of 12 genetic clusters identified by PCR fingerprinting, across 11 of 22 pathotypes differentiated by their pattern of compatibility with 11 resistance genes and combinations thereof, and across 17 of the 18 rice-growing states of the country. Molecular characterisation of resistance-breaking strains is important for management and can inform our understanding of the disease. We thus became interested in probing the basis for the *xa13* breakdown in India. The authors of the 400 isolate study kindly provided an *xa13*-breaking isolate from Haryana designated IX-221, of interest for being compatible with multiple resistance genes. We aimed, in particular, to characterise the major TALE(s) of IX-221.

We began by sequencing the genome, as described (Booher et al. 2015; Carpenter et al. 2020). Assembly yielded a complete, 4.9 Mb chromosome and no plasmids (Tables S2, S8). There are 20 encoded TALEs, of which 18 fall into existing AnnoTALE classes (v. 1.5; Grau et al. 2016) (Tables S3, S9), including a truncTALE (also 'iTALE'), a type of TALE with shortened N- and C-terminal regions that suppresses resistance mediated by *Xo1* or *Xa1* (Ji et al. 2016; Read et al. 2016). The number and sizes of *TALE* genes were confirmed by Southern blot (Figure S1). Two were classified as new by AnnoTALE. Of these, one contains only five RVDs and is thus likely non-functional. The other is orthologous with PthXo3, based on FuncTALE analysis, which groups TALEs by predicted targets (Pérez-Quintero et al. 2015). Strikingly, IX-221 also harbours two distinct orthologues of PthXo2. No orthologue of any other major TALE is present.

The PthXo3 orthologue, $Tal2b_{IX\text{-}221}$ (AnnoTALE class IU), like PthXo3, has a 39-aa repeat that in PthXo3 disengages for target binding (Richter et al. 2014). However, $Tal2b_{IX\text{-}221}$ has minor RVD sequence differences from PthXo3, different from those in two other variants (Oliva et al. 2019). $Tal2b_{IX\text{-}221}$ is thus the fourth member of the PthXo3 family and is hereafter referred to as $PthXo3D_{IX\text{-}221}$. With the full RVD sequence of $PthXo3D_{IX\text{-}221}$ as input, using TALE-NT 2.0 (Doyle et al. 2012) and the *japonica* rice cv. Nipponbare sequence, no binding in the *SWEET14* promoter was predicted; but with the RVD of the 39-aa repeat excluded, $PthXo3D_{IX\text{-}221}$ was predicted to bind at the PthXo3 EBE (Figure S2). As expected, IX-221 and the *pthXo1* mutant ME2 of Xoo strain PXO99A (Yang and White 2004) expressing a $pthXo3D_{IX\text{-}221}$ clone, each induced *SWEET14* in Nipponbare leaves (Figure S3, Tables S4, S10). Interestingly, aligned to the Nipponbare EBE, PthXo3 has a better binding score ratio (Doyle et al. 2012) than $PthXo3D_{IX\text{-}221}$ (Figure S2). This is due to $PthXo3D_{IX\text{-}221}$ containing more RVDs with relaxed base specificity: three NS RVDs, which accommodate A, C or G, and one NN, which recognises G or A. That flexibility may allow activation of yet uncharacterised *SWEET14* alleles that vary at those positions in the EBE. The flexibility may in fact have been selected for by such alleles.

The PthXo2 orthologues are $Tal6b_{IX\text{-}221}$ and $Tal7_{IX\text{-}221}$. PthXo2 variants reported to date include PthXo2B and PthXo2C (Oliva et al. 2019). While PthXo2 has standard, 34-aa repeats throughout, PthXo2B and 2C both have 36-aa 9th and 12th repeats and differ at a few RVDs from PthXo2. $Tal6b_{IX\text{-}221}$ is a novel variant, with just one 36-aa repeat, number 12. Alignment of the RVD sequences puts Tal6b between PthXo2 and PthXo2B and PthXo2C together (Figure S4). We hereafter refer to $Tal6b_{IX\text{-}221}$ as $PthXo2D_{IX\text{-}221}$.

$Tal7_{IX\text{-}221}$ is identical to PthXo2B from strain PXO61 ($PthXo2B_{PXO61}$), with 36-aa 9th and 12th repeats (Oliva et al. 2019; Xu et al. 2019) except that, relative to $Tal7_{IX\text{-}221}$, $PthXo2B_{PXO61}$ has glycine inserted at position 33, in the N-terminal region associated with type III secretion and dispensable for DNA binding (Szurek et al. 2002; Miller et al. 2011), and a 2-aa substitution, Q31R and D32A, in repeat 19. While D and A are common at position 32 in TALE repeats, R at position 31 is unusual. We hereafter refer to $Tal7_{IX\text{-}221}$ as $PthXo2B_{IX\text{-}221}$.

$PthXo2D_{IX\text{-}221}$ and $PthXo2B_{IX\text{-}221}$ seem unnecessary alongside *SWEET14* activator $PthXo3D_{IX\text{-}221}$, so we asked whether these PthXo2 variants in fact activate *SWEET13*. Beginning with $PthXo2D_{IX\text{-}221}$, we first determined the binding score ratio for its RVD sequence on the *SWEET13* allele present in the *indica* variety IR24 and the allele in Nipponbare, using TALE-NT 2.0. Because the 36-aa repeat type is capable of disengaging

(Becker et al. 2022), we also determined the score ratios using the sequence with that RVD excluded. Based on the score ratios, PthXo2D$_{IX\text{-}221}$ is expected to bind well to the IR24 allele with its 36-aa repeat engaged, and marginally to the Nipponbare allele with it excluded (Figure 1a).

Next, to test the predictions, we cloned the native *pthXo2D*$_{IX\text{-}221}$ CRR (as an SphI fragment) between the N- and C-terminal regions of Tal1c from *X. oryzae* pv. *oryzicola* (Xoc) strain BLS256 in the expression vector pKEB31 (Cermak et al. 2011) and assembled a designer TALE (dTALE) construct encoding PthXo2D$_{IX\text{-}221}$ with its aberrant repeat replaced by a standard repeat, also using the Tal1c context (Figure S5). We designated these constructs PthXo2D and dPthXo2D_34, respectively. To control for any effect of differences in the dTALE repeat backbone sequences from the native ones, we also generated a dTALE equivalent of native PthXo2D$_{IX\text{-}221}$, that is, with its 36-aa 12th repeat, dPthXo2D_36. We tested each of these constructs in ME2 inoculated to IR24 and Nipponbare leaves. ME2 expressing PthXo2D and ME2 expressing dPthXo2D_36 each induced *SWEET13* and caused long lesions when inoculated to IR24 (Figure 1b–d). In Nipponbare, neither detectably induced *SWEET13* nor caused lesions typical of a fully virulent strain. However, ME2 with PthXo2D elicited lesions longer than the negative control, ME2 with pKEB31 (encoding Tal1c with no CRR) (Figure 1b,c). dPthXo2D_34, with the standard repeat, behaved the same as PthXo2, inducing *SWEET13* and restoring virulence to ME2 only in IR24, with no virulence increase in Nipponbare relative to the control. Altogether, the results suggest that PthXo2D$_{IX\text{-}221}$ is functionally distinct from PthXo2, conferring virulence not just in *indica* rice but also weakly in *japonica* rice.

As a complementary approach and to confirm binding, we used *Agrobacterium*-mediated transient transformation in *Nicotiana benthamiana*, as described (Römer et al. 2009), to

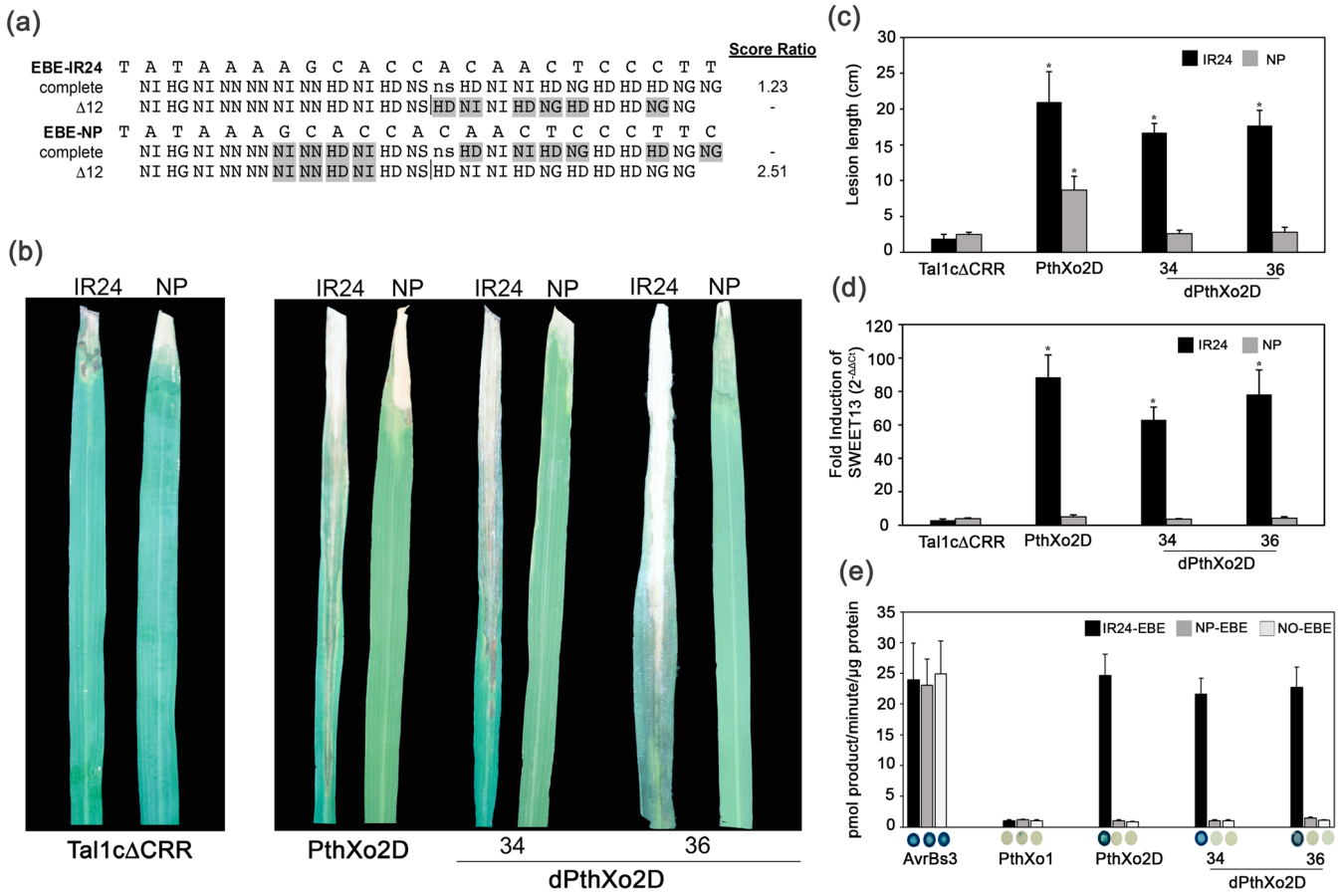

FIGURE 1 | PthXo2D$_{IX\text{-}221}$ acts as a major TALE activating *SWEET13* in IR24 without its 36-amino acid (aa) repeat disengaging. (a) TALE-NT 2.0-predicted binding of PthXo2D$_{IX\text{-}221}$, and a variant with the aberrant repeat removed, to *SWEET13* alleles of Nipponbare (NP) and IR24. Grey highlights repeat-variable diresidues (RVDs) mismatching the aligned base. The RVD of the 36-aa repeat is lowercase. Its omission is indicated by a vertical line. A lower score ratio indicates a higher probability of binding. A '–' indicates a score ratio above the cut-off of 3 used to predict binding. (b) Representative images and (c) lesion lengths on leaves of 6-week-old plants 14 days after clip inoculation with ME2 expressing the indicated TALE, with Tal1c lacking the central repeat region (CRR) (Tal1cΔCRR) as negative control. (d) Expression of *SWEET13*, measured by reverse transcription-quantitative PCR, 24–27 h following syringe infiltration of leaves with ME2 expressing the indicated TALE or control, relative to mock-inoculum. Error bars represent standard deviation ($n = 3$). Asterisks in (c) and (d) denote difference (Student's t test, $p < 0.01$) from the Tal1cΔCRR control in that variety. (e) Reporter assay of effector-specific binding element (EBE) binding by PthXo2D and dTALE variants as indicated. *Nicotiana benthamiana* leaves were co-infiltrated with *Agrobacterium tumefaciens* strains delivering the indicated TALE or dTALE construct and a β-glucuronidase (GUS) reporter driven by a minimal *Bs3* promoter containing the indicated effector-specific binding element (EBE), and activity assayed 48 h later. Representative leaf discs are shown.

test whether PthXo2D, dPthXo2D_34 and dPthXo2D_36 activate β-glucuronidase (GUS) reporter constructs driven by a minimal promoter from the pepper *Bs3* gene (Römer et al. 2009) amended with the PthXo2 *SWEET13* EBE from IR24 or with the sequence from Nipponbare. The TALE AvrBs3, which activates *Bs3*, was the positive control, and PthXo1, which has no EBE in either reporter, was the negative. PthXo2D, dPthXo2D_34 and dPthXo2D_36 each strongly induced the reporter harbouring the IR24 EBE and not the Nipponbare EBE (Figure 1e), validating the observations made in rice and confirming that $PthXo2D_{IX\text{-}221}$ binds the *SWEET13* allele in IR24 with its 36-aa repeat engaged. Why PthXo2D partially rescued ME2 in Nipponbare while dPthXo2D_36 did not is unclear.

For $PthXo2B_{IX\text{-}221}$, we followed the same approach. TALE-NT 2.0 predicted it to bind only the *japonica* allele, and only when the RVD of one or the other of its 36-aa repeats is excluded (Figure 2a). For virulence and *SWEET* induction assays (Figure 2b–d), similar to $PthXo2D_{IX\text{-}221}$, the expression constructs for $PthXo2B_{IX\text{-}221}$ included, in the context of Tal1c, the native CRR, a dTALE equivalent with 36-aa 9th and 12th repeats, and dTALEs with one, the other or both aberrant repeats converted to standard: PthXo2B, dPthXo2B_36_36, dPthXo2B_34_36, dPthXo2B_36_34 and dPthXo2B_34_34, respectively (Figure S5). PthXo2B in ME2, as predicted, induced *SWEET13* and restored virulence in Nipponbare and did not upregulate *SWEET13* in IR24 (Figure 2b–d). The dTALEs in ME2 revealed that activation of *SWEET13* in Nipponbare depends on one or the other of the 36-aa repeats disengaging: dPthXo2B_36_36, dPthXo2B_34_36 and dPthXo2B36_34 each activated *SWEET13* and restored virulence to ME2 in Nipponbare, while the dTALE with both repeats replaced, dPthXo2B_34_34, did not. Also, like PthXo2B, none of the dTALEs induced *SWEET13* in IR24. The binding assay results (Figure 2e) aligned with these observations, with all but dPthXo2B_34_34 strongly inducing the GUS reporter with the Nipponbare EBE and none inducing the reporter with the IR24 sequence, confirming that $PthXo2B_{IX\text{-}221}$ binds only the Nipponbare allele and that it relies on either of its two aberrant repeats disengaging to do so. This conclusion is consistent with that of Becker et al. (2022) using synthetic TalBK2 (AnnoTALE class for PthXo2B) and variants missing either repeat.

Surprisingly, despite PthXo2B not activating *SWEET13* in IR24, it nonetheless fully restored virulence to ME2 in that variety (Figure 2b,c), and it did so without activating any other clade III *SWEET* gene (Figure 2f). Consistent with the latter observation, PthXo2B has no predicted binding site in any other clade III *SWEET* promoter, in either orientation. This result suggests the involvement of a non-clade III *SWEET* susceptibility target. Further exploration is necessary to test this hypothesis. The ability of $PthXo2B_{IX\text{-}221}$ to function as a major TALE in an *indica* rice genotype strikingly differentiates it from $PthXo2B_{PXO61}$, as reported by Xu et al. (2019) and Oliva et al. (2019), despite the two having the same sequence of RVDs: $PthXo2B_{IX\text{-}221}$ in those studies did not restore virulence to ME2 in IR24. In addition to the -33G insertion in $PthXo2B_{PXO61}$ noted above, $PthXo2B_{PXO61}$ has some substitutions relative to the fragment of Tal1c used for the $PthXo2B_{IX\text{-}221}$ expression construct (and the dTALEs), likewise in the region associated with type III secretion and dispensable for DNA binding (Szurek et al. 2002; Miller et al. 2011). We infer that they are inconsequential and that instead the non-canonical substitution at repeat 19 is responsible for the difference in activity. We speculate that the substitution changes the specificity or affinity contribution of repeat 19, altering the overall targeting profile such that $PthXo2B_{PXO61}$ does not activate any alternative S gene. Another, not mutually exclusive, possibility is that the substitution makes repeat 19 like an aberrant one but disengages in an obligate rather than facultative fashion. Finally, it is formally possible that the difference is due to a lower amount of $PthXo2B_{PXO61}$ in the plant cell because that study used the low copy plasmid pHM1 for $PthXo2B_{PXO61}$ while we used the moderate copy number plasmid pKEB31.

More surprising still, the $PthXo2B_{IX\text{-}221}$-equivalent dTALE dPthXo2B_36_36 failed to restore virulence to ME2 in IR24, just as the $PthXo2D_{IX\text{-}221}$ dTALE equivalent dPthXo2D_36 failed to restore any virulence in Nipponbare, while PthXo2D, with the native CRR, partially did so. Alignment of the respective CRRs and flanking regions bordered by the SphI sites used for cloning (Figure S6) revealed some differences. Outside the CRR, there are a few substitutions in the native proteins relative to the dTALEs, which we presume to be inconsequential. Within the CRR, the standard repeat consensus subsequence VAIAS present in the dTALEs is replaced by MAIAN in the native CRRs, in repeats harbouring the RVD NN (repeats 4 and 6 of $PthXo2B_{IX\text{-}221}$ and 4, 5 and 7 of $PthXo2D_{IX\text{-}221}$). And, in the 6th repeat of $PthXo2B_{IX\text{-}221}$ and the 7th of $PthXo2D_{IX\text{-}221}$, the canonical D or A at position 4 is replaced by T. To explore the prevalence of these substitutions, we scanned TALE repeat sequences from randomly picked Xoo as well as Xoc genomes. The NN repeats of all PthXo2 orthologues, from diverse Xoo strains, have the MAIAN subsequence substitution for VAIAS, and no other repeats in those orthologues or any repeats in other TALEs do (Tables S5, S11). Similarly, all PthXo2 orthologues have T at position 4, and no other TALEs do. Further, each of the two subsequences is present in TALEs of Xoc (Tables S5, S11), always in an NN repeat, but never together in the same repeat. We hypothesise that the 'MAIAN' and 'T' substitutions in $PthXo2B_{IX\text{-}221}$ relative to the dTALEs are important for its ability to act as a major TALE in IR24 and that these substitutions in $PthXo2D_{IX\text{-}221}$ are important for its ability to confer some virulence in Nipponbare. Presumably, similar to the substitution in repeat 19, these substitutions impact the targeting profiles such that the native proteins induce one or more S genes other than a clade III *SWEET*. Whether this is the case and how, remains to be explored.

In summary, we have shown that $PthXo2B_{IX\text{-}221}$ activates *SWEET13* in *japonica*, and ostensibly an alternative S gene in *indica*, rendering genotypes in both subspecies susceptible. Unfortunately, because the dTALE equivalent of $PthXo2B_{IX\text{-}221}$ did not render ME2 virulent in IR24, we were unable to test with our standard repeat derivatives whether the aberrant repeats are important in *indica*. RVD sequences with one, the other, neither or both excluded should be used for binding site prediction in future pursuit of the putative alternative S gene. The potential existence of a non-clade III *SWEET* major S gene for bacterial blight is significant. The central roles of *SWEET11*, *SWEET13* and *SWEET14* in rice bacterial blight have inspired the development of lines of select *indica* and *japonica* mega-varieties edited at EBEs in all three to provide broad-spectrum bacterial blight resistance (Xu et al. 2019; Oliva et al. 2019). Our results predict that strains

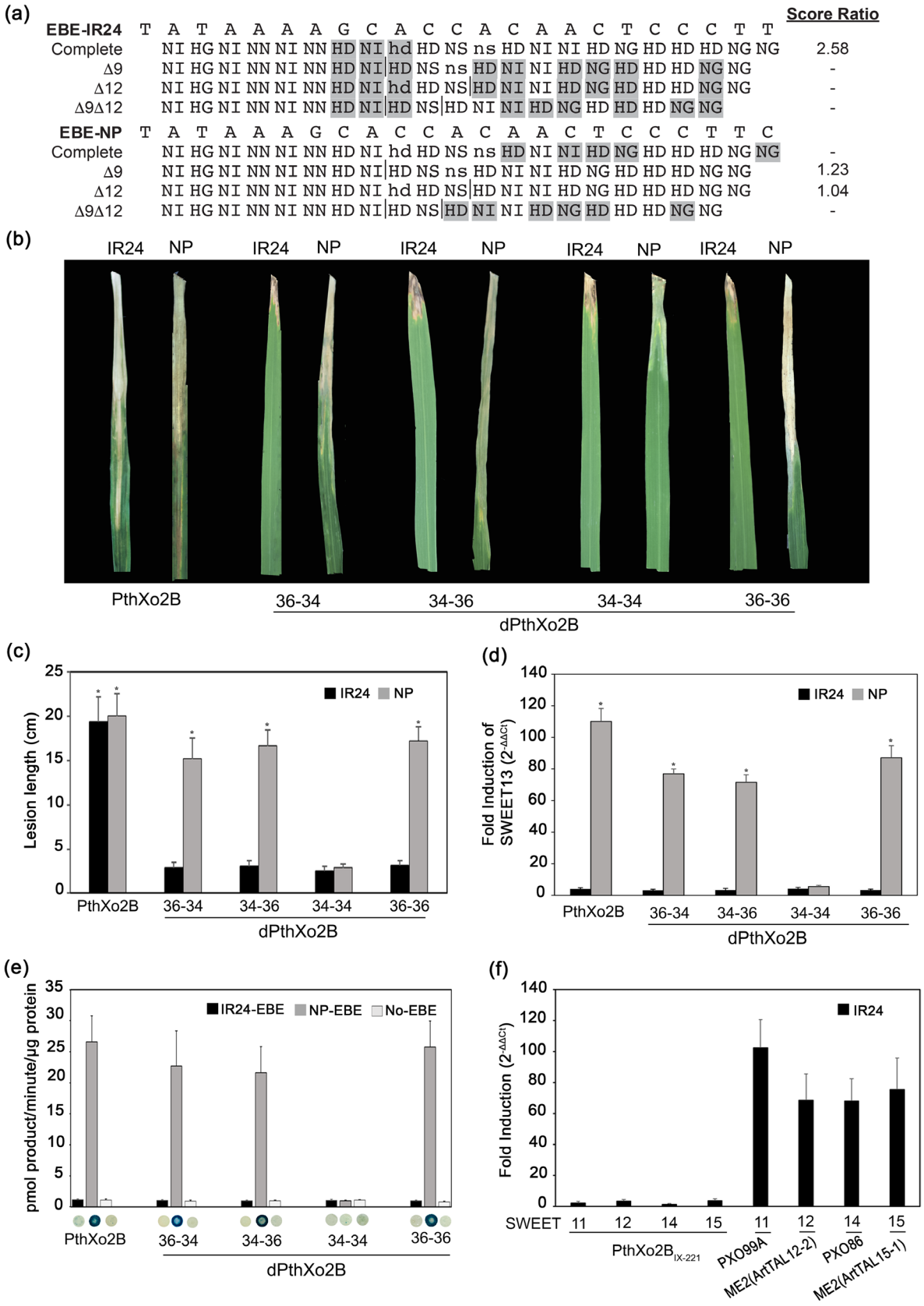

FIGURE 2 | PthXo2B$_{IX-221}$ acts as a major TALE in IR24 without activating any clade III *SWEET* and in Nipponbare by activating *SWEET13* with either of its 36-amino acid repeats disengaging. TALE-NT 2.0-predicted binding of PthXo2B$_{IX-221}$ and variants with one or both aberrant repeats removed, to *SWEET13* alleles of NP and IR24, as in Figure 1. (b–e) as in Figure 1 with PthXo2B and dTALE variants as indicated. Asterisks in (c) and (d) denote differences from control, shown in Figure 1. (f) Fold induction, as in (b), of the other clade III *SWEET* genes by PthXo2B and positive control TALEs.

harbouring PthXo2B$_{IX-221}$ will circumvent this resistance. In pointing to an alternative S gene, our findings also open the door to better mechanistic understanding of the disease.

Altogether, the presence of three major TALEs in IX-221, each with one or more repeat types that can facultatively disengage and one with more lax DNA targeting specificity than its previously characterised orthologue, provides compelling evidence that the widespread deployment of *xa13* has exerted intense selection pressure on Xoo populations in India to acquire or evolve TALEs that break it. Of note, while PthXo2B in each of four additional Philippine strains (Oliva et al. 2019) has the same Q31R substitution in repeat 19 that PthXo2B$_{PXO61}$ does, we found a PthXo2B allele in the Taiwanese strain XM9 that matches PthXo2B$_{IX-221}$, suggesting that the ability to activate the putative alternative S gene is not restricted to Indian strains. Furthermore, given the distribution of *xa13* compatibility across genotypes, pathotypes and locations in India (Yugander et al. 2017), there seem likely to be additional adaptations that break *xa13* in novel ways.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Genome data for IX-221 generated in this study are available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/ as accession CP019228.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.