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Improved bacterial leaf blight disease resistance in the major elite Vietnamese rice cultivar TBR225 via editing of the OsSWEET14 promoter

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Abstract:	TBR225 is one of the most popular commercial rice varieties in Northern Vietnam. However, this variety is very susceptible to bacterial leaf blight (BLB), a disease caused by Xanthomonas oryzae pv. oryzae (Xoo) which inflicts important yield losses. OsSWEET14 belongs to the SWEET gene family that encodes sugar transporters. Together with other Clade III members, it behaves as a susceptibility (S) gene whose induction by Asian Xoo Transcription-Activator-Like Effectors (TALEs) is absolutely necessary for disease. In this study, we sought to introduce BLB resistance in the TBR225 elite variety. First, two Vietnamese Xoo strains were shown to up-regulate OsSWEET14 upon TBR225 infection. To investigate if this induction is connected with disease susceptibility, nine TBR225 mutant lines with mutations in the AvrXa7, PthXo3 or TalF TALEs DNA target sequences of the OsSWEET14 promoter were obtained using the CRISPR/Cas9 editing system. Genotyping analysis of T 0 and T 1 individuals showed that mutations were stably inherited. None of the examined agronomic traits of three transgene-free T2 edited lines were significantly different from those of wild-type TBR225. Importantly, one of these T 2 lines, harboring the largest homozygous 6-bp deletion, displayed decreased OsSWEET14 expression as well as a significantly reduced susceptibility to a Vietnamese Xoo strains and complete resistance to the other one. Our finding indicated that CRISPR/Cas9 is a useful and effective approach to improve BLB resistance of commercial elite rice varieties.
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3 # rice cultivar TBR225 via editing of the *OsSWEET14* promoter

4 **Short title:**

5 **Improved bacterial leaf blight disease resistance in Vietnamese rice cultivar**
6 **TBR225**

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Abstract

TBR225 is one of the most popular commercial rice varieties in Northern Vietnam. However, this variety is very susceptible to bacterial leaf blight (BLB), a disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) which inflicts important yield losses. *OsSWEET14* belongs to the *SWEET* gene family that encodes sugar transporters. Together with other Clade III members, it behaves as a susceptibility (*S*) gene whose induction by Asian *Xoo* Transcription-Activator-Like Effectors (TALEs) is absolutely necessary for disease. In this study, we sought to introduce BLB resistance in the TBR225 elite variety. First, two Vietnamese *Xoo* strains were shown to up-regulate *OsSWEET14* upon TBR225 infection. To investigate if this induction is connected with disease susceptibility, nine TBR225 mutant lines with mutations in the AvrXa7, PthXo3 or TalF TALEs DNA target sequences of the *OsSWEET14* promoter were obtained using the CRISPR/Cas9 editing system. Genotyping analysis of T_0 and T_1 individuals showed that mutations were stably inherited. None of the examined agronomic traits of three transgene-free T2 edited lines were significantly different from those of wild-type TBR225. Importantly, one of these T_2 lines, harboring the largest homozygous 6-bp deletion, displayed decreased *OsSWEET14* expression as well as a significantly reduced susceptibility to a Vietnamese *Xoo* strains and complete resistance to the other one. Our finding indicated that CRISPR/Cas9 is a useful and effective approach to improve BLB resistance of commercial elite rice varieties.

41 ***Keywords**: **Bacterial leaf blight; CRISPR/Cas9; Xanthomonas oryzae*** **pv.** ***oryzae;***

42 ***OsSWEET14; TBR225; transgene-free plants.***

43

Introduction

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a major bacterial disease that causes 10%-20% annual reduction in rice production worldwide [1]. The use of improved rice varieties resistant to *Xoo* is probably the most efficient, economical and environmentally-friendly way to control BLB.

The virulence of *Xoo* depends on the transcriptional activation of specific host disease-susceptibility (*S*) genes by a subgroup of bacterial type III effectors, called transcription activator-like effectors (TALEs) [2]. Upon translocation into the plant cell, TALEs bind to specific host nuclear gene promoter sequences termed Effector-Binding Elements (EBEs) and induce target gene expression to the benefit of the pathogen. The central repetitive domain of TALEs is responsible for DNA target sequence binding. DNA binding involves recognition principles that have been largely deciphered and applied to the computational prediction of TALEs target DNA sequences [3,4]. This and earlier work has fostered the identification of TALEs transcriptional targets in the rice genome and ultimately, of rice BLB *S* genes [2].

All *Xoo* strains recurrently target *S* genes belonging to the *SWEET* gene family and coding for transmembrane sugar exporter proteins [3]. The over accumulation of SWEETs due to TALE induction is presumed to provide an additional ration of apoplastic carbohydrates for full bacterial pathogen multiplication and disease expression [5]. Although all five rice clade III *SWEET* genes can function as *S* genes for bacterial

64 blight, only three, namely *OsSWEET11*, *OsSWEET13* and *OsSWEET14*, are known to be
65 targeted by several unrelated TALEs in nature [6–11]. *OsSWEET11* is activated by
66 PthXo1 [6], *OsSWEET13* is targeted by different variants of PthXo2 [11,12], while
67 *OsSWEET14* is a target of multiple TAL effectors, including AvrXa7, PthXo3, TalC and
68 TalF [7–9].

69 Previous studies established that rice resistance to *Xoo* resulting from "TALE-
70 unresponsive" alleles can be conferred by natural DNA polymorphisms or targeted
71 editions in EBEs located in *OsSWEET* genes promoters of rice germplasm accessions or
72 engineered rice varieties, respectively [6,13–16]. For example, early resistance
73 engineering work has used TALENs to individually alter the AvrXa7, TalC or TalF EBEs
74 in the *OsSWEET14* promoter and successfully obtained resistance to some Asian *Xoo*
75 strains [13,15]. However, strains collected in Asian countries such as China, Japan,
76 Phillippines, Taiwan, Thailand, India, Nepal or South Korea can express combinations of
77 up to three major TALEs redundantly targeting clade III *OsSWEET* genes with either
78 PthXo3 or AvrXa7 being occasionally associated with PthXo2 [11,17]. Broad BLB
79 resistance engineering thus required multiplex *OsSWEET* promoters EBE editing using
80 the CRISPR/Cas9 system [11,12].

81 The clustered regularly interspaced short palindromic repeats/CRISPR-associated
82 protein-9 nuclease (CRISPR/Cas9) system is a simple and efficient gene-editing tool
83 developed in the past few years. Moreover, the targeted mutations generated by
84 CRISPR/Cas9 can be stably transmitted to the next generation. Thus, CRISPR/Cas9 has

85 become a routine tool in plant laboratories around the world to create various mutants for
86 many applications, including the genetic improvement of crops [18].

87 BLB is a major rice disease which occurs in many rice cultivating areas of Vietnam
88 [19,20]. Most Vietnamese commercial rice varieties, including TBR225, are susceptible
89 to BLB, resulting in annual yield loss of about 15 – 30% on average [21]. A few studies
90 have identified rice resistance genes effective against Vietnamese *Xoo* lineages [20,21].
91 However, no information is currently available on the nature of Vietnamese *Xoo* TALEs
92 and their corresponding *S* genes. Despite the large number of mapped rice BLB resistance
93 genes [22], there is a need for alternative breeding approaches that enable the rapid
94 introduction of broad BLB resistance in elite varieties in order to cope with swift
95 pathogen populations adaptive shifts in the fields [11,23].

96 Here, we report on the identification of *OsSWEET14* as a transcriptional target of
97 Vietnamese *Xoo*. CRISPR/Cas9-mediated mutagenesis of the *OsSWEET14* promoter in
98 TBR225, a major elite variety in rice production areas of North Vietnam is shown to
99 confer BLB resistance without detectable yield penalty. This study found this
100 quintessential *S* gene to be associated with the virulence of Vietnamese *Xoo* strains. This
101 is an important step for the future design and implementation of broad-spectrum BLB-
102 resistant in elite rice varieties using genome editing in Vietnam.

103

104 # Materials and methods

105 ## Plant and pathogen materials

106 Rice cultivar TBR225 (*Oryza sativa* L. ssp. *indica*) were obtained from ThaiBinh Seed
107 Cor. [24]. All edited and wild-type (WT) TBR225 plants were grown in a net-house
108 under the following average conditions: 30°C for 14 h (light) and 25°C for 10 h (dark)
109 with 80% humidity. The *Xoo* VXO_11 and VXO_15 strains used in this study were
110 isolated from diseased leaves collected in Hanoi-Vietnam in 2013 and 2016, respectively.
111 Bacteria were cultured as described in Zhou et al. (2015) [25].

112 ## Gene expression analysis

113 Gene expression analyses were carried out as described previously [26] by RT-PCR
114 method. The rice leaves were infiltrated with the indicated bacterial strains and used for
115 total RNA extraction 48 h post inoculation using the TRIzol reagent (Invitrogen, USA).
116 One microgram of RNA was used for each RT-PCR with oligo (dT) primer followed by
117 PCR with *OsSWEET14*-specific primers (forward 5′-
118 ACTTGCAAGCAAGAACAGTAGT-3′ and reverse 5′-
119 ATGTTGCCTAGGAGACCAAAGG-3′). An *Eppendorf Mastercycler ep Gradient S* was
120 used for 35 PCR cycles. The *OsEF1α* gene was used as a constitutive control [15] using
121 specific primers (forward 5′-GAAGTCTCATCCTACCTGAAGAAG-3′ and reverse 5′-
122 GTCAAGAGCCTCAAGCAAGG-3′).

123 ## gRNA design

124 The *OsSWEET14* promoter (GenBank, accession number: AP014967.1) was amplified by
125 PCR with forward primer 5'-TTGCGGCTCATCAGTTTCTC-3' and reverse primer 5'-
126 CTAGGAGACCAAAGGCGAAG-3' from genomic DNA of TBR225 rice plants and
127 ligated in pGEM-T Easy vector (Promega) for sequencing. The gRNA target sequence
128 (Fig 1A) for editing the TBR225 *OsSWEET14* promoter was designed based on the
129 sequence of the cloned TBR225 *OsSWEET14* promoter using a combination of two
130 bioinformatics tools CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/) and CCTop
131 (https://crispr.cos.uni-heidelberg.de/). A gRNA sequence with high on-target and low off-
132 target scores in both prediction tools was chosen for vector construction.

133

134 **Fig 1. CRISPR/Cas9-induced *OsSWEET14* promoter modification in TBR225 rice.**
135 (A) A region of the *OsSWEET14* promoter containing four EBEs (TalC, PthXo3, AvrXa7
136 and TalF) and putative TATA box from TBR225. The target site (complementary to the
137 guide RNA) is shown in the box, immediately following the protospacer adjacent motif
138 (PAM). (B) T-DNA region of the CRISPR/Cas9-mediated genome editing construct
139 carrying OsSWEET14-sgRNA (indicated by the black box). The expression of *Cas9* is
140 driven by the maize *ubiquitin* promoter (P-Ubi); the expression of the OsSWEET14-
141 sgRNA is driven by the rice *OsU6* promoter (P-OsU6a); the expression of *HPT* is driven
142 by two *CaMV35S* promoters (P-2×35S); T-35S, T-Nos and TTTTTT: gene terminators;
143 LB and RB: left and right border, respectively. (C) Alignment of the *OsSWEET14*
144 promoter fragment in the nine T_0 transgenic TBR225 rice plants edited in the AvrXa7,

145 PthXo3 and TalF EBEs. The lines on top of the wild-type sequence represent the binding
146 sites of AvrXa7, PthXo3 and TalF. The arrow indicates the expected cutting site of the
147 Cas9 complex used in this study. The labels on the left indicate the name of examined
148 mutant lines; (a1) and (a2) distinguish alleles in the same line. The numbers on the right
149 indicate the type of mutation and the number of nucleotides involved; (+) and (-) indicate
150 insertion and deletion, respectively.

151

152 **Vector construction**

153 The Cas9 rice expression vector (pUbi-Cas9) [27] and the sgRNA expression vector
154 (pENTR-sgRNA) under the control of the *OsU6* promoter [28] were used to construct the
155 pCas9/OsSWEET14-gRNA expression vector. The complementary oligonucleotides with
156 appropriate 4-bp overhangs were synthesized by Macrogen (Korea). After heat
157 denaturation, the complementary oligonucleotides (5′-
158 gtgtGGTGCTAAGCTCATCAAGCC-3’ and 5′-aaacGGCTTGATGAGCTTAGCACC-
159 3’) were first annealed to each other, phosphorylated, and ligated into the *Bsa*I-digested
160 vector pENTR-sgRNA. The integrity of the inserted fragment was verified by
161 sequencing. Subsequently, the sgRNA cassette was cloned into pUbi-Cas9 using the
162 Gateway LR clonase (Life Technologies) (Fig 1B). The resulting construct was
163 confirmed by Sanger sequencing of the insertion junctions.

164

165 ## *Agrobacterium*-mediated rice transformation

166 The pCas9-OsSWEET14-gRNA was electroporated into *Agrobacterium tumefaciens*
167 EHA105 and the resulting strain was used to transform rice using the method described
168 by Hiei et al. (1994) [29]. The presence of the transgene in the genome of T_0
169 hygromycin-resistant plants or segregating T_1 individuals was evaluated by PCR using 5′-
170 ATGGCCCCAAAGAAGAAG-3′ and 5′- GCCTCGGCTGTCTCGCCA-3′ primers
171 specific for *Cas9*. T1 individuals were analyzed by PCR using *Cas9*, *OsSWEET14-gRNA*
172 (5′- GGATCATGAACCAACG-3′ and 5′- GAATTCGATATCAAGCTT-3′) and *HPT*
173 (5’-AAACTGTGATGGACGACACCGT-3’ and 5′- GTGGCGATCCTGCAAGCTCC -
174 3′) specific diagnostic primer pairs together with a positive control pair (5′-
175 TTGCGGCTCATCAGTTTCTC-3′ and 5′- TGGATCAGATCAAAGGCAAC -3′)
176 specific to the *OsSWEET14* promoter.

177

178 ## Bacterial blight inoculation

179 Rice cultivation and disease assays were done according to the methods of Blanvillain-
180 Baufumé et al. (2017) [15]. Bacteria were cultured in PSA media (10 g/liter peptone, 10
181 g/liter sucrose, 1 g/liter glutamic acid, 15 g/liter Bacto Agar) at 28°C for two days [30]
182 and inoculated at an optical density (OD_{600}) of 0.5 (infiltrations) or 0.4 (leaf clipping) in
183 water. For lesion length measurements, at least three inoculated leaves per plant and three
184 plants for each line were measured 14 days after inoculation (DAI), and scored as

185 follows: high resistance (lesion length < 8 cm), moderate resistance (lesion length 8-12
186 cm) and susceptibility (lesion length > 12 cm). For gene expression analyses, 4-cm leaf
187 sections which were infiltrated with bacterial suspensions were collected at 48 h after
188 inoculation for RNA extraction. Experiments included samples from three pooled
189 biological replicate leaves. The plants inoculated with distilled water only were used as
190 negative controls.

191

192 ## Analysis of *OsSWEET14* edited allele sequences

193 To determine the nature of the mutation at the target site, all transgenic T_0 or T_1 plants
194 were analyzed by PCR using genomic DNA (50 ng) as a template and *OsSWEET14*
195 specific primers (5′-TTGCGGCTCATCAGTTTCTC-3′ and 5′-
196 TGGATCAGATCAAAGGCAAC -3′). The PCR products were directly sequenced using
197 the Sanger method. The sequencing chromatograms were decoded using the Degenerate
198 Sequence Decoding method [31] in order to identify the mutations.

199

200 ## Evaluation of major agronomic traits under net-house conditions

201 WT and selected mutant plants were planted under net-house conditions in a randomized
202 pot design experiment. At maturity, five plants of each line were investigated for the
203 following agronomic traits: growth duration, plant height, number of tillers per plant,
204 number of grains per panicle, number of filled grains per panicle and yield (seed mass)

per plant. The experiment was repeated three times, so a total of fifteen plants were evaluated for each line.

Analysis of potential off-target editing

Off-target sequences were predicted with the CCTop tool (https://crispr.cos.uni-heidelberg.de) **against the** *OsSWEET14* promoter **sgRNA** and the rice Nipponbare genome with default parameters. A total of 18 potential off-target sequences were identified. Three of them were located in coding regions (Table S2). These regions were amplified by PCR using the specific primers listed in Table S2 and analyzed by sequencing.

Results

Vietnamese *Xoo* strains induce *OsSWEET14* during infection of the TBR225 rice variety

OsSWEET14/Os11N3 was previously identified as a susceptibility gene for *Xoo* strains relying on either of the AvrXa7, PthXo3, TalF (formerly Tal5) or TalC TALEs for infection of the rice cultivars Nipponbare and Kitaake [11]. Because *Xoo* strains tend to frequently target this gene, we first sequenced a region of the *OsSWEET14* promoter from rice cultivar TBR225 to examine if it also carries documented target EBEs. Based

224 on the Nipponbare genome sequence in database (AP014967.1), the region encompassing
225 1343 bp sequence upstream and 52 bp sequence downstream of the predicted
226 transcription start site of *OsSWEET14* gene from TRB225 rice cultivar was PCR
227 amplified and sequenced (S1 Fig). The promoter region including the putative TATA box
228 (TATAAA) and the AvrXa7, PthXo3, TalF/Tal5 and TalC EBEs (Fig 1A), located 319
229 bp to 216 bp upstream of the ATG initiation codon, showed 100% identity to the
230 Nipponbare sequence. This therefore implied that in principle, the TBR225 *OsSWEET14*
231 promoter can be recognized by characterized major *Xoo* TALEs.

232 As illustrated by the representative experiment of Fig 2A, we also challenged TBR225
233 plants with two Vietnamese *Xoo* strains VXO_11 and VXO_15, both originating from the
234 Hanoi province, using leaf clipping assays. We consistently obtained typical extended
235 disease lesions 14 days after inoculation (25.5 cm and 26.6 cm average lesions length for
236 VXO_11 and VXO_15, respectively in the experiment of S2 Fig), indicating that the
237 TBR225 variety is susceptible to BLB.

238 To test if *OsSWEET14* is a potential direct virulence target of Vietnamese *Xoo* strains, we
239 infiltrated TBR225 rice leaves with the two Vietnamese *Xoo* strains. Forty-eight hours
240 post infiltration, TBR225 plants inoculated with VXO strains displayed a strong
241 induction of *OsSWEET14* relative to water controls (Fig 2B). These results suggest that
242 *OsSWEET14* is a transcriptional target of VXO strains and that it may act as a
243 susceptibility gene in TBR225.

244

245 **Fig 2. *OsSWEET14* is likely a susceptibility gene for Vietnamese *Xoo* strains in rice**
246 **cultivar TBR225**. (A) Representative images of the disease lesions obtained 14 days
247 after leaf clipping inoculation of TBR225 rice leaves with Vietnamese *Xoo* strains
248 VXO_11 and VXO_15 or with water (CT). The chevrons above the leaves indicate the
249 maximum visible extent of lesions away from the inoculation point on the left (B).
250 *OsSWEET14* expression pattern obtained by RT-PCR two day post-infiltration of
251 TBR225 rice leaves with Vietnamese *Xoo* strains. CT Plants were inoculated with water
252 only. The experiment was repeated three times.

253

254 ## CRISPR/Cas9 design for *OsSWEET14* promoter editing

255 Our main objective was to engineer resistance to BLB caused by Vietnamese *Xoo* strains.
256 To this end, we subsequently sought to specifically modify the *OsSWEET14* promoter in
257 TBR225 rice with CRISPR/Cas9-mediated editing. Previous work revealed that while
258 African *Xoo* strains rely on TalC and occasionally, TalF, all Asian *Xoo* strains use either
259 PthXo3- or AvrXa7-like TALEs to activate *OsSWEET14* [11]. Because the *talC* gene is
260 currently exclusively found in African strains, we reasoned that it is unlikely that
261 Vietnamese strains carry a *talC* copy. Thus, to maximize our chances to perturb all
262 remaining documented EBEs, we selected a 20-bp nucleotide target site overlapping the
263 PthXo3, AvrXa7 and TalF EBEs and having a predicted cut site located near the 3'-end of
264 the AvrXa7 EBE (Fig 1A). The recombinant binary plasmid pCas9/OsSWEET14-gRNA
265 for CRISPR/Cas9 mediated editing of *OsSWEET14* was transformed into the rice variety

266 TBR225 via *Agrobacterium*-mediated transformation (S1 Table). A total of nine TBR225
267 transformants were selected from 10 independent PCR-validated transgenic T_0 TBR225
268 plants to further investigate CRISPR/Cas9-targeted mutagenesis of the *OsSWEET14*
269 promoter. In order to decipher the nature of the editing events in *OsSWEET14*, the
270 promoter sequencing data of transgenic lines were analyzed using the Degenerate
271 Sequence Decoding software [31]. All 9 T_0 transgenic plants harbored at least an edition
272 event (Fig 1C): two were heterozygous mutant/wild type, two had homozygous
273 mutations, and five had bi-allelic mutations. Regarding the type of mutations, 66.7%
274 were nucleotide deletions, 11.1% of the mutations were nucleotide insertions and no
275 substitution was detected (Table 1).

276

277 **Table 1. Frequencies of mutant genotypes and target mutation types in T_0**
278 **transgenic plants.**

Mutant genotype ratios[a] (%)			Mutation type ratios[b] (%)		
Heterozygote	**Homozygote**	**Bi-allelic**	**Deletion**	**Insertion**	**Substitution**
22.2 (2/9)	22.2 (2/9)	55.6 (5/9)	66.7 (12/18)	11.1 (2/18)	0 (0/18)

[a] (Number of on-target mutant genotype/total number of on-target mutant genotypes) x 100%.
[b] (Number of allele mutation type/number of all allele mutation types) x 100%.

279

280 Inheritance of CRISPR/Cas9-induced mutations in the T_1 281 generation

282 To assess the inheritance of the CRISPR/Cas9-induced *OsSWEET14* mutations in the
283 next generation, all T_0 mutant transgenic plants (Fig 1C) were allowed to self-pollinate,
284 and T_1 transgenic plants were randomly selected in the progeny of T_0 plants for
285 sequencing and analysis of their edited site (Table 2). All T_1 individuals derived from T_0
286 plants previously genotyped as homozygous possessed the same allele as their parent,
287 suggesting stable inheritance of the mutations to the next generation. Similarly, the T1
288 progeny of each of both bi-allelic and heterozygous mutation T_0 lines showed a
289 segregation ratio which is consistent with Mendelian segregation ($\chi^2 < \chi^2_{0.05,\ 2} = 5.99$),
290 indicating that the CRISPR/Cas9-induced mutations in T_0 plants were transmitted as
291 expected to the next generation. Interestingly, no new mutant allele was detected in the T_1
292 generation of both heterozygous mutants L-21 and L-27, even though most of them still
293 carried the transgene. Overall, consistent with previous similar studies, our results
294 indicate that the CRISPR/Cas9-mediated mutations generated here are stably transmitted
295 to the next generation in a Medelian fashion.

296 **Table 2. Transmission of CRISPR/Cas9 editing events to the T_1 generation.**

T_0 plant	Genotype	Allele(s)	No. of T_1 plants tested	Mutation inheritance in the T_1 generation		No. of T-DNA-free plants
				Alleles segregation	χ^2 (1:2:1)	
L-4	Bi-allelic	-5/-3	32	10 (-5), 18 (-5/-3), 4 (-3)	2,750	5 (2*)
L-5	Bi-allelic	-6/+1	44	9 (-6), 22 (-6/+1), 13 (+1)	0,727	10 (2*)
L-7	Bi-allelic	-4/-3	38	14 (-4), 17 (-4/-3), 7 (-3)	3,000	11 (4*)

L-15	Homozygote	+1	5	5 (+1)	-	1 (1*)
L-21	Heterozygote	-3	26	3 (-3), 13 (-3/wt), 10 (wt)	3,769	7 (1*)
L-27	Bi-allelic	-5/-4	7	1 (-5), 3(-5/-4), 3 (-4)	1,286	0
L-29	Heterozygote	-5	33	6 (-5), 19 (-5/wt), 8 (wt)	1,000	2 (0*)
L-31	Homozygote	-3	15	15 (-3)	-	5 (5*)
L-54	Bi-allelic	-3/-2	21	3 (-3), 12 (-3/-2), 6(-2)	1,286	3 (0*)

"+" and "-" indicate respectively, insertion and deletion, of the indicated number of nucleotides.
"w", wild type.
*Number of homozygous mutant plants without T-DNA.

Selection of transgene-free mutant TBR225 rice lines

To identify T-DNA free T_1 rice plants containing a mutation in EBEs of the *OsSWEET14* promoter, PCR analysis was carried out using primers specific to *Cas9*, sgRNA and *HPT* sequences (Table 2). A T1 individual was considered devoid of the transgene if the control amplification of the *OsSWEET14* promoter was successful and if none of the PCR reactions with independent primer pairs designed on the T-DNA produced a detectable diagnostic band. The results of this PCR screen show that the T-DNA could be segregated out in the progeny of most T_0 lines, with 88.9% of the T_0 lines generating T-DNA-free progeny. In total, 44 of 221 analyzed edited T_1 plants did not generate a specific amplicon from the T-DNA construct and 15 of them were homozygous mutant harboring the desired *OsSWEET14* modifications. Our results demonstrate that transgene-free, homozygous mutant individuals could be obtained in the segregating progeny of selfed T_0 individuals.

311

312 **TBR225 *OsSWEET14* promoter editing confers resistance to**
313 **Vietnamese *Xoo***

314 To characterize the BLB-resistance phenotype of the generated rice mutants, three T-
315 DNA-free, homozygous TBR225 edited lines, namely, L-5.7(-6), L-31.12(-3) and L-
316 15.4(+1) with *OsSWEET14* promoter alleles corresponding respectively to L-5-a1 (6bp
317 deletion), L-31 (3bp deletion) and L-15 (1bp insertion) in Fig 1C, were established.
318 Selected T_1 individuals were propagated to obtain T_2 seeds which were used to perform
319 BLB susceptibility assays. Edited T_2 and WT TBR225 plants were inoculated by leaf-
320 clipping with the VXO_11 and VXO_15 strains at the eight-week stage. The inoculated
321 leaves of wild type TBR225 plants and of edited lines L-15.4(+1) and L-31.12(-3)
322 developed long water-soaked lesions typical of BLB, ranging from 18.3 cm to 29.0 cm in
323 length. In contrast, the edited line L-5.7(-6), harboring a longer 6-bp deletion at the target
324 site, displayed high (1.2 cm average lesion length) and moderate (7.3 cm average lesion
325 length) resistance to VXO_11 and VXO_15 strains, respectively (Fig 3). Means
326 comparisons with a Tukey's HSD test further indicated that irrespective of the inoculated
327 strain, the mean lesion lengths measured on the L-15.4(+1), L-31.12(-3) or wild type
328 lines were not significantly different. In contrast, the mean lesion lengths recorded on the
329 L-5.7(-6) mutant line were significantly different from those obtained on the wild type
330 and the two other edited lines challenged with either of the Vietnamese strains (Fig 3B).
331 Furthermore, our off-target editing analysis on line L-5.7(-6) did not reveal unintended

332 modifications of other annotated rice loci (Table S2 and Figure S5), indicating that the 6-
333 bp deletion in the *OsSWEET14* promoter is probably responsible for this phenotype.

334 Consistent with disease assays and as shown in Figure 3C, whereas a semiquantitative
335 RT-PCR signal for *OsSWEET14* expression was detected on the parental variety and the
336 L-15.4(+1) and L-31.12(-3) edited lines following VXO_11 and VXO_15 infiltration,
337 this amplicon was undetectable in the resistant L-5.7(-6) line.

338 In conclusion, this data shows that the 6-bp deletion in the AvrXa7/PthXo3 EBE reduces
339 dramatically *OsSWEET14* expression following VXO strains inoculation and confers
340 resistance to these strains. In contrast, shorter modifications on the 3'-end of this EBE are
341 insufficient to perturb *OsSWEET14* expression after inoculation and do not confer
342 detectable protection against the corresponding strains. Finally, while these results
343 strongly support the view that *OsSWEET14* functions as a unique susceptibility gene in
344 the interaction between strain VXO_11 and the TBR225 rice variety, the resistance to
345 strain VXO_15 is not as dramatic and may suggest that other mechanisms partially
346 counteract the effects of the AvrXa7/PthXo3 EBE 6-bp deletion in edited TBR225 plants.

347

348 **Fig 3. BLB resistance assays for homozygous mutant rice lines L-5.7(-6), L-15.4(+1)**
349 **and L-31.12(-3)**. (A) Leaves were photographed 14 days post-leaf clipping inoculation of
350 *Xoo* strains VXO_11 and VXO_15; arrow heads indicate the end of the lesion. (B) Mean
351 lesion lengths (bars) and standard deviations (error bars). Values were measured 14 days
352 post-leaf clipping inoculation of two *Xoo* strains VXO_11 and VXO_15 and were

353 computed from at least three leaves from each of three plants. Asterisks indicate
354 significant differences relative to wild type plants (Tukey's HSD test; $^{**}P < 0.05$). The
355 number in the parentheses following the line name indicates the type of mutation and the
356 number of nucleotides involved. The letters above strain labels indicate susceptibility
357 score (R - high resistance; M – moderate resistance; S - susceptibility). The experiment
358 was repeated three times. (C) *OsSWEET14* expression pattern obtained by RT-PCR two
359 day post-infiltration of genome edited homozygous mutant rice lines L-31.12(-3), L-
360 15.4(+1) and L-5.7(-6) and parental TBR225 rice leaves with Vietnamese *Xoo* strains.
361 This experiment was repeated two times with similar results.

362

363 TBR225 *OsSWEET14* promoter edited lines agronomic 364 performances are undistinguishable from the parental variety

365 To determine if mutations in the *OsSWEET14* promoter affect agronomic traits of
366 TRB225 rice plants, three independent homozygous mutant lines were analyzed by
367 measuring their growth duration, plant height, number of tillers per plant, number of
368 grains per panicle, number of filled grains per panicle, yield per plant and amylose
369 content under net-house conditions (see picture of S3 Fig). ANOVA tests and Student's *t*
370 tests showed that the mutant lines displayed no significant difference to TBR225, in
371 terms of the examined agronomic traits, under our net-house conditions (Table 3). These

372 results suggest that the tested CRISPR/Cas9-induced mutations in the *OsSWEET14*
373 promoter did not negatively impact the main agronomic traits of TBR225.

374

375 **Table 3. Agronomic traits evaluation of homozygous T_2 mutant lines.**

Lines	Growth duration (day)	Plant height (cm)	No. of tillers per plant	No. of grains per panicle	No. of filled grains per panicle	Amylose content (%)
WT	108.4 ± 1.1[a]	86.6 ± 3.2[a]	5 ± 0.7[a]	144.4 ± 4.9[a]	125 ± 4.5[a]	13.2 ± 0.38[a]
L-5.7(-6)	108 ± 1.2[a]	86.4 ± 4.3[a]	5.2 ± 0.4[a]	144.2 ± 4.4[a]	123.4 ± 5.5[a]	13.7 ± 0.35[a]
L-15.4(+1)	107.8 ± 0.8[a]	86.4 ± 5.0[a]	4.8 ± 0.4[a]	147.8 ± 5.1[a]	121.8 ± 3.0[a]	13.5 ± 0.41[a]
L-31.12(-3)	108 ± 1.2[a]	88.4 ± 4.3[a]	5.4 ± 0.5[a]	144.6 ± 5.3[a]	124.2 ± 7.4[a]	13.8 ± 0.21[a]

Five plants per line were measured. Experiments were repeated three time.
Means followed by the same letter do not differ significantly ($P < 0.05$).

376

377

378 # Discussion

379 Recently, the CRISPR/Cas9 system has emerged as a powerful tool for gene editing in
380 many organisms including plants. Because of its specificity and efficiency, this system
381 has been widely used to improve important agronomic traits of major crops such as rape,
382 tomato, soybean, rice, wheat and maize [32]. Excluding easy-to-transform reference
383 accessions such as Nipponbare and Kitaake that are widely used in the laboratory, the
384 number of reports on the improvement of agriculturally relevant elite rice cultivars for
385 pertinent traits using the CRISPR/Cas9 technology (see for example [33–36]) is gradually
386 increasing but is still limited.

387 TBR225 [24], a major commercial rice variety cultivated in large areas of Northern
388 Vietnam, has the advantages of early maturity, high and stable yield, as well as cooking
389 quality. However, it is very susceptible to BLB. Here, the CRISPR/Cas9-mediated
390 editing method was applied in order to rapidly improve the BLB resistance of TBR225 by
391 modifying the AvrXa7, PthXo3 and TalF EBEs on the promoter of *OsSWEET14*. Of the
392 three generated homozygous mutant lines tested for resistance, the one carrying the
393 largest deletion at the target site (6 bp) showed a significantly improved resistance to
394 infection with two *Xoo* strains VXO_11 and VXO_15. Therefore, using the major
395 commercial rice variety TBR225 as an example, we illustrate the advantages of
396 CRISPR/Cas9 tool for rice breeding.

397 In the present study, the frequency of individuals with CRISPR/Cas9-induced mutations
398 in T_0 transgenic plants was 90%, which is similar to previous observation [28]. We
399 obtained only two heterozygous mutant/wild type lines versus seven homozygous or bi-
400 allelic mutant lines. This high frequency of mutated alleles is another proof that the
401 CRISPR/Cas9 system is indeed an efficient tool for gene editing in plant. We also
402 observed the stable transmission of edited alleles to subsequent generations. This is a
403 common phenomenon that has been repeatedly documented for rice plants carrying
404 CRISPR/Cas9-induced mutations [33,35]. In this study, we obtained only two types of
405 induced mutations in T_0 plants: insertion (11.1%) and deletion (66.7%), but no
406 substitution were observed. In some earlier studies, new mutations were continuously
407 obtained in the T_1 offspring of heterozygous T_0 mutants because the Cas9 complex

408 remains active on edited targets until the seed or PAM regions cease to be functional
409 [35,37,38]. In contrast, here, all the T_1 plants generated from both heterozygous lines L-
410 21 and L-29, regardless of whether they had a CRISPR/Cas9 T-DNA transgene
411 integrated in their genome, did not show any new mutation possibly because
412 CRISPR/Cas9 T-DNA transgene was no longer functional. We could also readily obtain
413 transgene-free plants from most of the T_1 segregation populations without any laborious
414 crossing or backcrossing steps, which illustrates an advantage of the CRISPR/Cas9
415 technology compared to conventional breeding.

416 Clade III SWEET family proteins are involved in a number of biological processes such
417 as seed and pollen development or pathogen susceptibility [39]. Their inactivation has
418 previously been shown to cause pleiotropic and/or detrimental effects. For example, both
419 *ossweet11* single and *ossweet11-ossweet15* double Kitaake rice mutants showed defects
420 in endosperm development and filling [40]. In addition, RNA-mediated silencing of
421 either *Os11N3/OsSWEET14* [7] or *Os8N3/OsSWEET11* [6] in BLB resistant Kitaake
422 lines causes negative effects on seed production. In contrast, here, we show that T-DNA-
423 free TBR225 plants harboring homozygous mutations generated with the CRISPR/Cas9
424 system in the AvrXa7/PthXo3 EBE of the *OsSWEET14* promoter exhibited enhanced
425 *Xoo* resistance but did not show any significant difference in all examined agronomic
426 traits compared to wild-type plants under net-house growth conditions. It is conceivable
427 that limited modifications in promoter regions do not affect the normal expression of
428 *SWEET* genes in contrast to KO or silenced lines. Our findings are consistent with the

429 previous work of Oliva et al. [11] who studied 30 combinations of EBE mutations in the
430 *OsSWEET11*, *OsSWEET13* and *OsSWEET14* promoters of the IR64 or Ciherang-Sub1
431 varieties and detected only a single line with abnormal agronomic traits.

432 Some individual *Xoo* strains have evolved a set of distinct TALE effectors that
433 collectively target several members of the clade III SWEET family. The presence of these
434 redundant TALEs thereby trumps single "loss-of-tale-responsiveness" resistance alleles
435 [11,12,17,41]. For example, Kitaake lines carrying TALEN-induced mutation in the
436 *SWEET14* promoter [13,15] exhibit resistance to strains which depend exclusively on
437 matching AvrXa7/PthXo3 for clade III *SWEET* family induction. Likewise, the natural
438 *xa13* allele [42] or CRISPR/Cas9-induced mutation in the *SWEET11* promoter [11]
439 exhibit resistance to strains such as PXO99 which depend exclusively on PthXo1, for
440 virulence. However, the BLB resistance of the Kitaake lines harboring mutations in both
441 AvrXa7/PthXo3 (*OsSWEET14*) and PthXo1 (*OsSWEET11*) EBEs was defeated by *Xoo*
442 strains expressing simultaneously the AvrXa7/PthXo3 and PthXo2B TALEs [11].
443 Recently, the stacking of EBE-edited alleles in several *OsSWEET* promoters have
444 overcome this limitation and was shown to achieve a broad spectrum of resistance to
445 strains from most BLB-prone countries in Asia [11,12].

446 All of the three T_2 lines tested for BLB resistance were affected for the AvrXa7/PthXo3
447 EBE and conserved an otherwise wild type TalF EBE (Fig 1C). The homozygous mutant
448 TBR225 line L-5.7(-6) carrying a 6-bp deletion in the AvrXa7/PthXo3 EBE exhibited a
449 significantly enhanced resistance to two Vietnamese *Xoo* strains compared to WT

450 TBR225. The L-15.4(+1) and L-31.12(-3) lines that harbored more subtle alterations in
451 the 3'-end of this EBE (a 1-bp insertion and a 3-bp deletion, respectively) in contrast
452 remained susceptible to VXO strains. Our *OsSWEET14* expression analysis after
453 Vietnamese Xoo strains inoculation (Fig 1C) suggests that these editing events did not
454 alter the EBE sequence sufficiently to compromise promoter recognition by an
455 AvrXa7/PthXo3-like Vietnamese TALE. With less than 2 cm average lesion length, the
456 resistance of line L-5.7(-6) (6-bp deletion) to the VXO_11 strain is rather extreme (versus
457 average lesion length of 20.1 cm on wild type plants). Moreover, in this line,
458 *OsSWEET14* expression following bacterial inoculation is strongly reduced relative the
459 parental line and the two other edited lines, which suggest that in this case, recognition by
460 an AvrXa7/PthXo3-like Vietnamese TALE is abrogated. Consistent with *OsSWEET14*
461 expression analysis and as shown in S4 Fig, the Talvez [43] target prediction scores for
462 AvrXa7 and PthXo3 on the *OsSWEET14* promoter L-5-a1 allele sequence of line L-5.7(-
463 6) are markedly lower than on the wild type promoter sequence. This is not the case
464 however for the edited alleles carried by lines L-15.4(+1) and L-31.12(-3) (respectively
465 L-15 and L-31 in S4 Fig) whose Talvez scores are identical or slightly lower than those
466 of the wild type promoter sequence.

467 The magnitude of the effect of the 6-bp deletion allele on susceptibility to VXO_11 is
468 comparable to the dramatic effect of previously characterized alterations of the same
469 EBEs in the Kitaake background against the PXO86 strain that possesses a single TALE,
470 AvrXa7, targeting *OsSWEET14* for clade III *OsSWEET* gene induction [15]. By analogy,

471 this suggests that *OsSWEET14* is also the only clade III *OsSWEETs* target of VXO_11 in
472 the TBR225 background but, in order to confirm this hypothesis an examination of other
473 clade III *OsSWEET* genes expression patterns in response to this strain would be
474 required. The situation with the VXO_15 strain is not as straightforward to interpret and
475 will require further investigations. Although the 6-bp deletion in the AvrXa7/PthXo3
476 EBE did provide an increased resistance to the edited plants, the VXO_15 strain caused
477 intermediate disease severity (7.3 cm average lesion length on Fig 3). This incomplete
478 resistance is unlikely to result from the partial but still productive recognition of
479 subsequences of the altered EBE by a VXO_15 AvrXa7/PthXo3-like TALE because
480 *OsSWEET14* expression is similarly decreased in response to either this strain or
481 VXO_11 (Fig 3C). Alternatively, contrary to all Asian *Xoo* examined so far, but similar
482 to African *Xoo* [15], VXO_15 may have the intrinsic potential to cause disease in the
483 absence of clade III *OsSWEET* gene induction. More likely, analogous to other Asian
484 strains, VXO_15 may encode alternative TALEs, such as PthXo2B or PthXo1 that
485 compensate the loss of *OsSWEET14* induction by targeting other clade III *OsSWEET*
486 genes. In this regard, long read genome sequencing will ultimately help describe TALEs
487 variability in Vietnamese *Xoo* strains.

488 In conclusion, we showed that editing specific EBEs of *Xoo* TALEs via CRISPR/Cas9
489 tool is an efficient method for improving BLB resistance of elite rice varieties such as
490 TBR225 without detectable yield penalties. This also uncovered the potential diversity of
491 TALEs in Vietnamese *Xoo* population, which will thus require future investigations to

address the TALE repertoires of Vietnamese *Xoo* strains in order to generate broad-spectrum BLB-resistant rice varieties in Vietnam.

Authors' Contributions

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520

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690

691 # Supporting information

692 **S1_raw_images. Original photograph used in Fig 2 for the RT-PCR gels panel.**

693 **S2_raw_images. Original photograph used in Fig 3C for the RT-PCR gels panel.**

694 **S1 Fig. Nucleotide sequence of the *OsSEET14* promoter in TBR225.**

695 **S2 Fig. Virulence of Vietnamese *Xoo* strains VXO_11 and VXO_15 on TBR225 rice.**
696 Grey points correspond to individual lesion length measurements while the black points
697 indicate the calculated average value. The line range represents standard deviation.

698 **S3 Fig. Picture of an individual plant from the homozygous mutant rice lines L-5.7(-**
699 **6).**

700 **S4 Fig. Talvez scoring of AvrXa7, PthXo3 and TalF target EBES in the edited**
701 ***OsSWEET14* promoter allele sequences.** Score values are represented both by the
702 length of the horizontal bar and a fill color scale. Higher Talvez prediction scores reflect
703 a better match between a predicted EBE and the sequence of RVD of the query TALE.

704 **S5 Fig. Amplicon sequencing of predicted off-target sites for the *OsSWEET14***
705 **promoter-sgRNA in annotated exons of the TBR225 edited line L-5.7(-6).** Potential
706 unintended target sequences including the PAM are highlighted in boxes. They are all
707 identical to the expected wild type Nipponbare sequences.

708 **S1 Table. Key figures on the TBR225 transformation procedure for *OsSWEET14***
709 **promoter editing.**

710 **S2 Table. Output of the CCTop tool used with the *OsSWEET14* promoter sgRNA**

711 **for off-target prediction on the rice Nipponbare genome.**

712

Fig1

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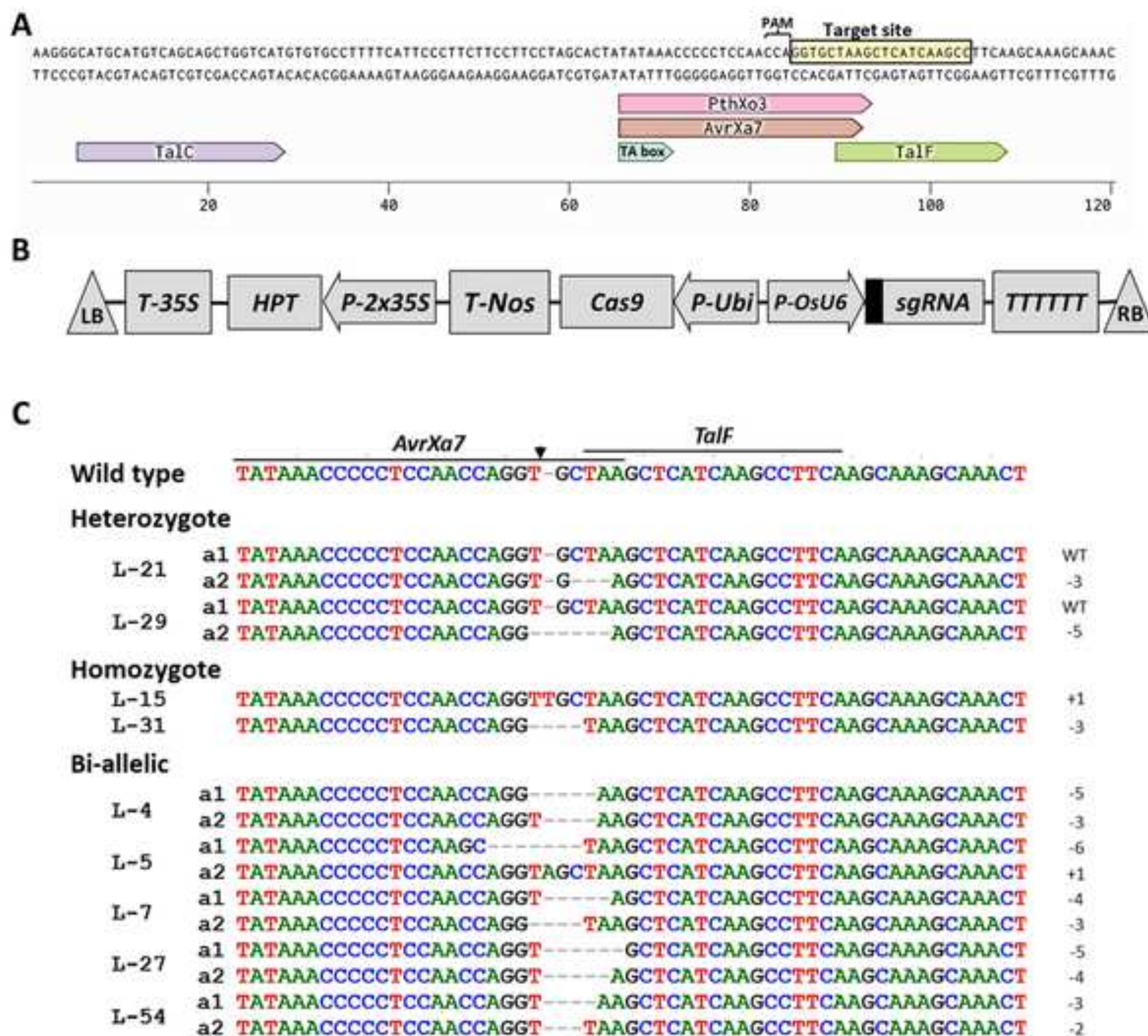

Fig2

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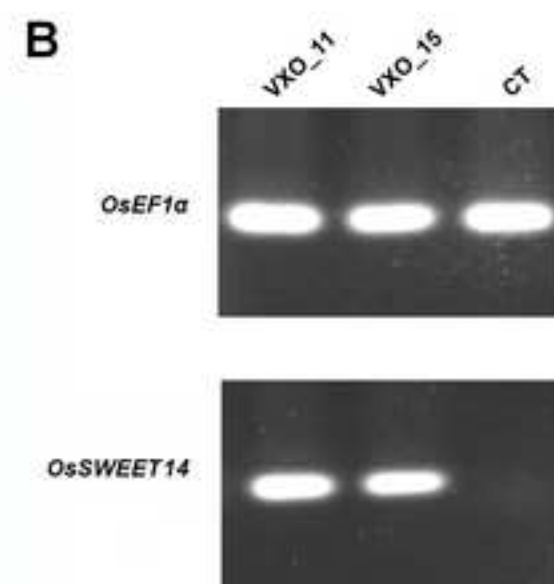

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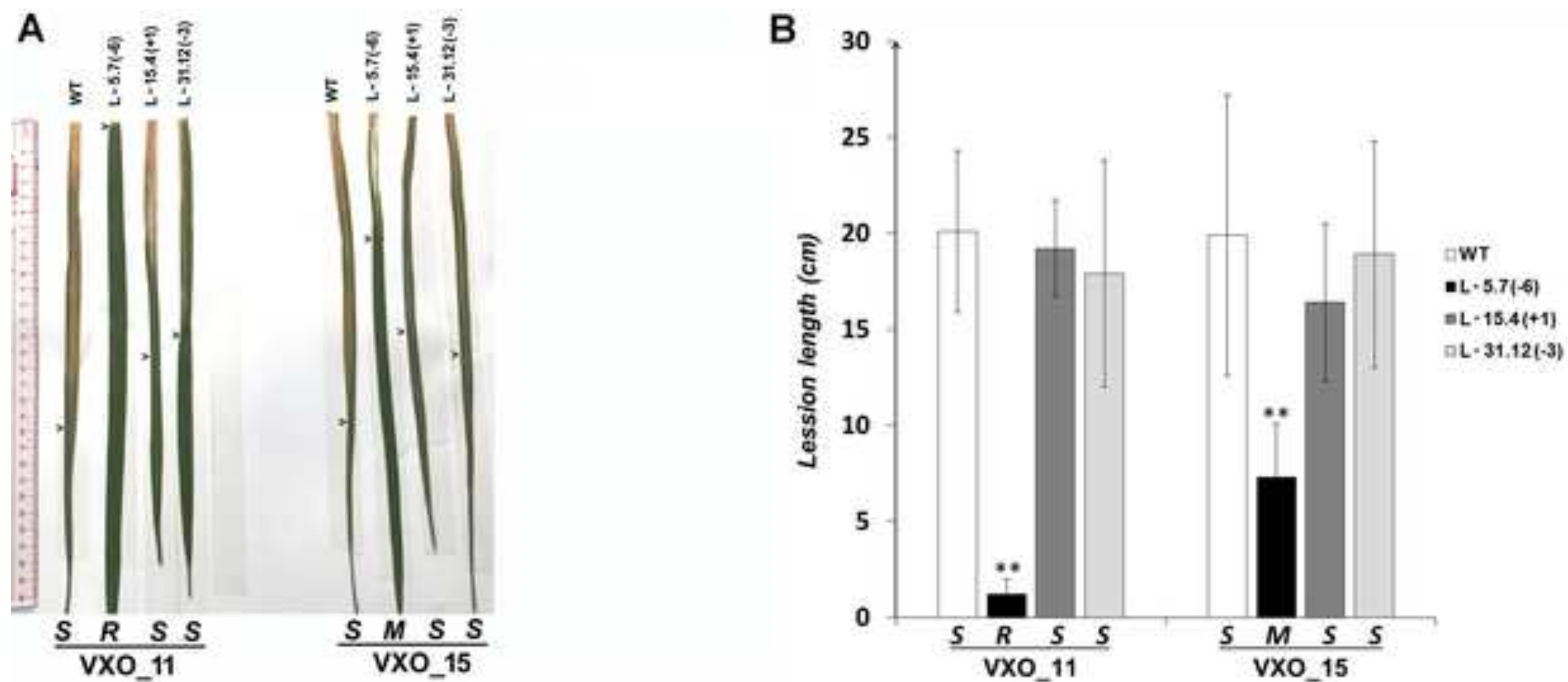

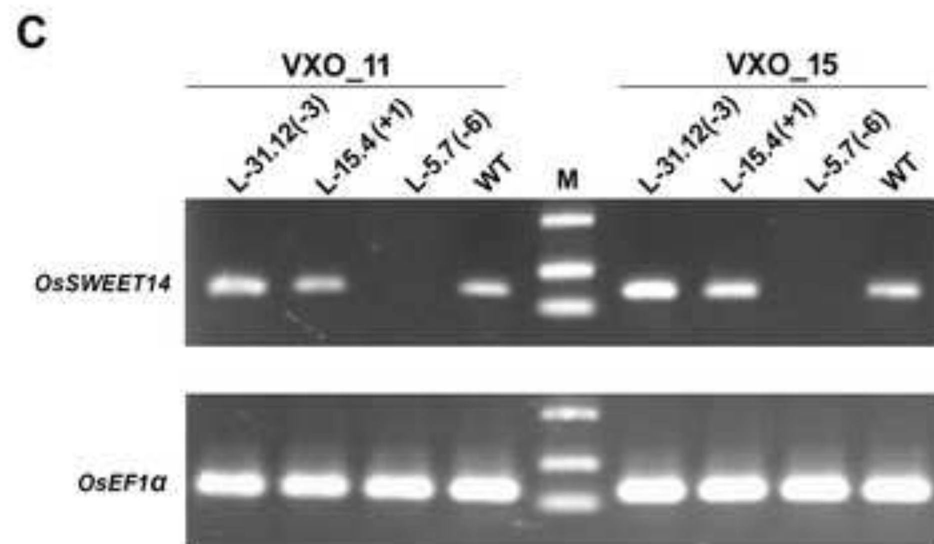

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1 **Full title:**

2 **Improved bacterial leaf blight disease resistance in the major elite Vietnamese**
3 **rice cultivar TBR225 via editing of the *OsSWEET14* promoter**

4 **Short title:**

5 **Improved bacterial leaf blight disease resistance in Vietnamese rice cultivar**
6 **TBR225**

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Abstract

TBR225 is one of the most popular commercial rice ~~variety~~varieties in Northern Vietnam. However, this variety is very susceptible to bacterial leaf blight (BLB), a disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) which inflicts important yield losses. *OsSWEET14* belongs to the *SWEET* ~~(*Sugars Will Eventually be Exported Transporter*)~~ gene family that encodes sugar transporters. Together with other Clade III members, it behaves as a susceptibility (*S*) gene whose induction by Asian *Xoo* Transcription-Activator-Like Effectors (TALEs) is absolutely necessary for disease. In this study, we sought to introduce BLB resistance in the TBR225 elite variety. First, two Vietnamese *Xoo* strains were shown to up-regulate *OsSWEET14* upon TBR225 infection. To investigate if this induction is connected with disease susceptibility, nine TBR225 mutant lines with mutations in the AvrXa7, PthXo3 or TalF TALEs DNA target sequences of the *OsSWEET14* promoter were obtained using the CRISPR/Cas9 editing system. Genotyping analysis of T_0 and T_1 individuals showed that mutations were stably inherited. None of the examined agronomic traits of three transgene-free T2 edited lines were significantly different from those of wild-type TBR225~~All examined agronomic traits of three transgene-free T_2 lines were not significantly different from those of wild-type TBR225~~. Importantly, one of these T_2 lines, harboring the largest homozygous 6-bp deletion, displayed decreased *OsSWEET14* expression as well as a significantly reduced susceptibility to ~~both~~ a Vietnamese *Xoo* strains and complete resistance to ~~one~~ the other

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41 oneof them. Our finding indicated that CRISPR/Cas9 is a useful and effective approach
42 to improve BLB resistance of commercial elite rice varieties.

43 ***Keywords**: **Bacterial leaf blight; CRISPR/Cas9; Xanthomonas oryzae*** **pv.** ***oryzae;***
44 ***OsSWEET14; TBR225; transgene-free plants.***

45

46 # Introduction

47 Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a major
48 bacterial disease that causes 10%-20% annual reduction in rice production worldwide [1].
49 The use of improved rice varieties resistant to *Xoo* is probably the most efficient,
50 economical and environmentally-friendly way to control BLB.

51 The virulence of *Xoo* depends on the transcriptional activation of specific host disease-
52 susceptibility (*S*) genes by a subgroup of bacterial type III effectors, called transcription
53 activator-like effectors (TALEs) [2]. Upon translocation into the plant cell, TALEs bind
54 to specific host nuclear gene promoter sequences termed Effector-Binding Elements
55 (EBEs) and induce target gene expression to the benefit of the pathogen. The central
56 repetitive domain of TALEs is responsible for DNA target sequence binding. DNA
57 binding involves recognition principles that have been largely deciphered and applied to
58 the computational prediction of TALEs target DNA sequences [3,—4]. This and earlier
59 work has fostered the identification of TALEs transcriptional targets in the rice genome
60 and ultimately, of rice BLB *S* genes [2].

61 All *Xoo* strains recurrently target *S* genes belonging to the *SWEET* gene family and
62 coding for transmembrane sugar exporter proteins [3]. The over accumulation of
63 SWEETs due to TALE induction is presumed to provide an additional ration of
64 apoplastic carbohydrates for full bacterial pathogen multiplication and disease expression
65 [5]. Although all five rice clade III *SWEET* genes can function as *S* genes for bacterial

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blight, only three, namely *OsSWEET11*, *OsSWEET13* and *OsSWEET14*, are known to be targeted by several unrelated TALEs in nature [6–11]. *OsSWEET11* is activated by PthXo1 [6], *OsSWEET13* is targeted by different variants of PthXo2 [11,~~–~~12], while *OsSWEET14* is a target of multiple TAL effectors, including AvrXa7, PthXo3, TalC and TalF [7–9].

Previous studies established that rice ~~recessive~~ resistance to *Xoo* resulting from "TALE-unresponsive" alleles can be conferred by natural DNA polymorphisms or targeted editions in EBEs located in *OsSWEET* genes promoters of rice germplasm accessions or engineered rice varieties, respectively [6,13–16]. For example, early resistance engineering work has used TALENs to individually alter the AvrXa7, TalC or TalF EBEs in the *OsSWEET14* promoter and successfully obtained resistance to some Asian *Xoo* strains [13,15]. However, ~~many~~ strains collected in Asian countries such as China, Japan, Phillippines, Taiwan, Thailand, India, Nepal or South Korea can express combinations of up to three major TALEs redundantly targeting clade III *OsSWEET* genes with either PthXo3 or AvrXa7 being ~~often~~ occasionally associated ~~to~~ with PthXo2 [11,17]. Broad BLB resistance engineering thus required multiplex *OsSWEET* promoters EBE editing using the CRISPR/Cas9 system [11,12].

The clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 nuclease (CRISPR/Cas9) system is a simple and efficient gene-editing tool developed in the past few years. Moreover, the targeted mutations generated by CRISPR/Cas9 can be ~~faithfully and~~ stably transmitted to the next generation. Thus,

CRISPR/Cas9 has become a routine tool in plant laboratories around the world to create various mutants for many applications, including the genetic improvement of crops [18].

BLB is a major rice disease which occurs in many rice cultivating areas of Vietnam [19,20]. Most Vietnamese commercial rice varieties, including TBR225, are susceptible to BLB, resulting in annual yield loss of about 15 – 30% on average [21]. A few studies have identified rice resistance genes effective against Vietnamese *Xoo* lineages [20,21]. However, no information is currently available on the nature of Vietnamese *Xoo* TALEs and their corresponding *S* genes.

Despite the large number of mapped rice BLB resistance genes [22], there is a need for alternative breeding approaches that enable the rapid introduction of broad BLB resistance in elite varieties in order to cope with swift pathogen populations adaptive shifts in the fields [11,23].

Here, we report on the identification of *OsSWEET14* as a transcriptional target of Vietnamese *Xoo*. CRISPR/Cas9-mediated mutagenesis of the *OsSWEET14* promoter in TBR225, a major elite variety in rice production areas of North Vietnam is shown to confer BLB resistance without detectable yield penalty. ~~For the first time, t~~This study found this quintessential *S* gene to be associated with the virulence of Vietnamese *Xoo* strains. This is an important step for the future design and implementation of broad-spectrum BLB-resistant in elite rice varieties using genome editing in Vietnam.

107 # Materials and methods

108 ## Plant and pathogen materials

109 Rice cultivar TBR225 (*Oryza sativa* L. ssp. *indica*) were obtained from ThaiBinh Seed
110 Cor. [24]. All edited and wild-type (WT) TBR225 plants were grown in a net-house
111 under the following average conditions: 30°C for 14 h (light) and 25°C for 10 h (dark)
112 with 80% humidity. The *Xoo* VXO_11 and VXO_15 strains used in this study were
113 isolated from diseased leaves collected in Hanoi-Vietnam in 2013 and 2016, respectively.
114 Bacteria were cultured as described in Zhou et al. (2015) [25].

115 ## Gene expression analysis

116 Gene expression analyses were carried out as described previously [26] by RT-PCR
117 method. The rice leaves were infiltrated with the indicated bacterial strains and used for
118 total RNA extraction 48 h post inoculation using the TRIzol reagent (Invitrogen, USA).
119 One microgram of RNA was used for each RT-PCR with oligo (dT) primer followed by
120 PCR with *OsSWEET14*-specific primers (forward 5′-
121 ACTTGCAAGCAAGAACAGTAGT-3′ and reverse 5′-
122 ATGTTGCCTAGGAGACCAAAGG-3′). An *Eppendorf Mastercycler ep Gradient S* was
123 used for 35 PCR cycles. The *OsEF1α* gene was used as a constitutive control [15] using
124 specific primers (forward 5′-GAAGTCTCATCCTACCTGAAGAAG-3′ and reverse 5′-
125 GTCAAGAGCCTCAAGCAAGG-3′).

gRNA design

The *OsSWEET14* promoter (GenBank, accession number: AP014967.1) was amplified by PCR with forward primer 5’-TTGCGGCTCATCAGTTTCTC-3’ and reverse primer 5’-CTAGGAGACCAAAGGCGAAG-3’ from genomic DNA of TBR225 rice plants and ligated in pGEM-T Easy vector (Promega) for sequencing. The gRNA target sequence (Fig 1A) for editing the TBR225 *OsSWEET14* promoter was designed based on the sequence of the cloned TBR225 *OsSWEET14* promoter using a combination of two bioinformatics tools CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/) and CCTop (https://crispr.cos.uni-heidelberg.de/). ~~The~~ A gRNA sequence with high on-target and low off-target scores in both prediction tools was chosen for vector construction.

Fig 1. CRISPR/Cas9-induced *OsSWEET14* promoter modification in TBR225 rice. (A) A region of the *OsSWEET14* promoter containing four EBEs (TalC, PthXo3, AvrXa7 and TalF) and putative TATA box from TBR225. The target site (complementary to the guide RNA) is shown in the box, immediately following the protospacer adjacent motif (PAM). (B) T-DNA region of the CRISPR/Cas9-mediated genome editing construct carrying OsSWEET14-sgRNA (indicated by the black box). The expression of *Cas9* is driven by the maize *ubiquitin* promoter (P-Ubi); the expression of the OsSWEET14-sgRNA is driven by the rice *OsU6* promoter (P-OsU6a); the expression of *HPT* is driven by two *CaMV35S* promoters (P-2×35S); T-35S, T-Nos and TTTTTT: gene terminators; LB and RB: left and right border, respectively. (C) Alignment of the *OsSWEET14*

147 promoter fragment in the nine T_0 transgenic TBR225 rice plants edited in the AvrXa7,
148 PthXo3 and TalF EBEs. The lines on top of the wild-type sequence represent the binding
149 sites of AvrXa7, PthXo3 and TalF. The arrow indicates the expected cutting site of the
150 Cas9 complex used in this study. The labels on the left indicate the name of examined
151 mutant lines; (a1) and (a2) distinguish alleles in the same line. The numbers on the right
152 indicate the type of mutation and the number of nucleotides involved; (+) and (-) indicate
153 insertion and deletion, respectively.

154

155 Vector construction

156 The Cas9 rice expression vector (pUbi-Cas9) [27] and the sgRNA expression vector
157 (pENTR-sgRNA) under the control of the *OsU6* promoter [28] were used to construct the
158 pCas9/OsSWEET14-gRNA expression vector. The complementary oligonucleotides with
159 appropriate 4-bp overhangs were synthesized by Macrogen (Korea). After heat
160 denaturation, the complementary oligonucleotides (5′-
161 gtgtGGTGCTAAGCTCATCAAGCC-3’ and 5′-aaacGGCTTGATGAGCTTAGCACC-
162 3’) were first annealed to each other, phosphorylated, and ligated into the *Bsa*I-digested
163 vector pENTR-sgRNA. The integrity of the inserted fragment was verified by
164 sequencing. Subsequently, the sgRNA cassette was cloned into pUbi-Cas9 using the
165 Gateway LR clonase (Life Technologies) (Fig 1B). The resulting construct was
166 confirmed by Sanger sequencing of the insertion junctions.

167

168 ## *Agrobacterium*-mediated rice transformation

169 The pCas9-OsSWEET14-gRNA was electroporated into *Agrobacterium tumefaciens*
170 EHA105 and the resulting strain was used to transform rice using the method described
171 by Hiei et al. (1994) [29]. The presence of the transgene in the genome of T_0
172 hygromycin-resistant plants or segregating T_1 individuals was evaluated by PCR using 5′-
173 ATGGCCCCAAAGAAGAAG-3′ and 5′- GCCTCGGCTGTCTCGCCA-3′ primers
174 specific for *Cas9*. T1 individuals were analyzed by PCR using *Cas9*, *OsSWEET14-gRNA*
175 (5′- GGATCATGAACCAACG-3′ and 5′- GAATTCGATATCAAGCTT-3′) and *HPT*
176 (5'-AAACTGTGATGGACGACACCGT-3' and 5′- GTGGCGATCCTGCAAGCTCC -
177 3′) specific diagnostic primer pairs together with a positive control pair (5′-
178 TTGCGGCTCATCAGTTTCTC-3′ and 5′- TGGATCAGATCAAAGGCAAC -3′)
179 specific to the *OsSWEET14* promoter.

180

181 ## Bacterial blight inoculation

182 Rice cultivation and disease assays were done according to the methods of Blanvillain-
183 Baufumé et al. (2017) [15]. Bacteria were cultured in PSA media (10 g/liter peptone, 10
184 g/liter sucrose, 1 g/liter glutamic acid, 15 g/liter Bacto Agar) at 28°C for two days [30]
185 and inoculated at an optical density (OD_{600}) of 0.5 (infiltrations) or 0.4 (leaf clipping) in
186 water. For lesion length measurements, at least three inoculated leaves per plant and three

plants for each line were measured 14 days after inoculation (DAI), and scored as follows: high resistance (lesion length < 8 cm), moderate resistance (lesion length 8-12 cm) and susceptibility (lesion length > 12 cm). For gene expression analyses, 4-cm leaf sections which were infiltrated with bacterial ~~solution~~ suspensions were collected at 48 h after inoculation for RNA extraction. Experiments included samples from three pooled biological replicate leaves. The plants inoculated with distilled water only were used as negative controls ~~in all experiments~~.

Analysis of *OsSWEET14* edited allele sequences

To determine the nature of the mutation at the target site, all transgenic T_0 or T_1 plants were analyzed by PCR using genomic DNA (50 ng) as a template and *OsSWEET14* specific primers (5′-TTGCGGCTCATCAGTTTCTC-3′ and 5′-TGGATCAGATCAAAGGCAAC -3′). The PCR products were directly sequenced using the Sanger method. The sequencing chromatograms were decoded using the Degenerate Sequence Decoding method [31] in order to identify the mutations.

Evaluation of major agronomic traits under net-house conditions

WT and selected mutant plants were planted under net-house conditions in a randomized pot design experiment. At maturity, five plants of each line were investigated for the following agronomic traits: growth duration, plant height, number of tillers per plant,

number of grains per panicle, number of filled grains per panicle and yield (seed mass) per plant. The experiment was repeated three times, so a total of fifteen plants were evaluated for each line.

Analysis of potential off-target ~~sequences~~editing

O~~O~~ff-target sequences were predicted~~,~~ ~~by~~with the ~~-~~CCTop tool~~s~~ (https://crispr.cos.uni-heidelberg.de~~/~~) **against the** *OsSWEET14* promoter ~~-~~**sgRNA** and the rice Nipponbare genome with default parameters. A total of 18 potential off-target sequences were identified. Three of them ~~, of which 3~~ were located in coding regions (Table S2). These regions were amplified by PCR using the specific primers listed in Table S2 and analyzed by sequencing.

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Results

Vietnamese *Xoo* strains induce *OsSWEET14* during infection of the TBR225 rice variety

OsSWEET14/Os11N3 was previously identified as a susceptibility gene for *Xoo* strains relying on either of the AvrXa7, PthXo3, TalF (formerly Tal5) or TalC TALEs for infection of the rice cultivars Nipponbare and Kitaake [11]. Because *Xoo* strains tend to frequently target this gene, we first sequenced ~~In this study,~~ a region of the *OsSWEET14*

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promoter from rice cultivar TBR225 ~~was sequenced~~ to examine if it also carries documented target EBEs. Based on the Nipponbare genome sequence in database (AP014967.1), the region encompassing 1343 bp sequence upstream and 52 bp sequence downstream of the predicted transcription start site of *OsSWEET14* gene from TRB225 rice cultivar was PCR amplified and sequenced (S1 Fig). The promoter region including the putative TATA box (TATAAA) and the AvrXa7, PthXo3, TalF/Tal5 and TalC EBEs (Fig 1A), located 319 bp to 216 bp upstream of the ATG initiation codon, showed 100% identity to the Nipponbare sequence. This therefore implied that in principle, the TBR225 *OsSWEET14* promoter can be recognized by characterized major *Xoo* TALEs.

As illustrated by the representative experiment of Fig 2A, we also challenged TBR225 plants with two Vietnamese *Xoo* strains VXO_11 and VXO_15, both originating from the Hanoi province, using leaf clipping assays. We consistently obtained typical extended disease lesions 14 days after inoculation (25.5 cm and 26.6 cm average lesions length for VXO_11 and VXO_15, respectively in the experiment of S2 Fig), indicating that the TBR225 variety is susceptible to BLB.

To test if *OsSWEET14* is a potential direct virulence target of Vietnamese *Xoo* strains, we infiltrated TBR225 rice leaves with the two Vietnamese *Xoo* strains. Forty-eight hours post infiltration, TBR225 plants inoculated with VXO strains displayed a strong induction of *OsSWEET14* relative to water controls (Fig 2B). These results suggest that *OsSWEET14* is a transcriptional target of VXO strains and that it may act as a susceptibility gene in TBR225.

247

248 **Fig 2. *OsSWEET14* is likely a susceptibility gene for Vietnamese *Xoo* strains in rice**
249 **cultivar TBR225**. (A) Representative images of the disease lesions obtained 14 days
250 after leaf clipping inoculation of TBR225 rice leaves with Vietnamese *Xoo* strains
251 VXO_11 and VXO_15 or with water (CT). The chevrons above the leaves indicate the
252 maximum visible extent of lesions away from the inoculation point on the left (B).
253 *OsSWEET14* expression pattern obtained by RT-PCR two day post-infiltration of
254 TBR225 rice leaves with Vietnamese *Xoo* strains. CT Plants were inoculated with water
255 only. The experiment was repeated three times.

256

257 CRISPR/Cas9 design for *OsSWEET14* promoter editing

258 Our main objective was to engineer resistance to BLB caused by Vietnamese *Xoo* strains.
259 To this end, we subsequently sought to specifically modify the *OsSWEET14* promoter in
260 TBR225 rice with CRISPR/Cas9-mediated editing. Previous work revealed that while
261 African *Xoo* strains rely on TalC and occasionally, TalF, all Asian *Xoo* strains use either
262 PthXo3- or AvrXa7-like TALEs to activate *OsSWEET14* [11]. Because the *talC* gene is
263 currently exclusively found in African strains, we reasoned that it is unlikely that
264 Vietnamese strains carry a *talC* copy. Thus, to maximize our chances to perturb all
265 remaining documented EBEs, we selected a 20-bp nucleotide target site overlapping the
266 PthXo3, AvrXa7 and TalF EBEs and having a predicted cut site located near the 3'-end of

267 the AvrXa7 EBE (Fig 1A). The recombinant binary plasmid pCas9/OsSWEET14-gRNA
268 for CRISPR/Cas9 mediated editing of *OsSWEET14* was transformed into the rice variety
269 TBR225 via *Agrobacterium*-mediated transformation (S1 Table). A total of nine TBR225
270 transformants were selected from 10 independent PCR-validated transgenic T_0 TBR225
271 plants to further investigate CRISPR/Cas9-targeted mutagenesis of the *OsSWEET14*
272 promoter. In order to decipher the nature of the editing events in *OsSWEET14*, the
273 promoter sequencing data of transgenic lines were analyzed using the Degenerate
274 Sequence Decoding software [31]. All 9 T_0 transgenic plants harbored at least an edition
275 event (Fig 1C): two were heterozygous mutant/wild type, two had homozygous
276 mutations, and five had bi-allelic mutations. Regarding the type of mutations, 66.7%
277 were nucleotide deletions, 11.1% of the mutations were nucleotide insertions and no
278 substitution was detected (Table 1).

279

280 **Table 1. Frequencies of mutant genotypes and target mutation types in T_0**
281 **transgenic plants.**

Mutant genotype ratios[a] (%)			Mutation type ratios[b] (%)		
Heterozygote	**Homozygote**	**Bi-allelic**	**Deletion**	**Insertion**	**Substitution**
22.2 (2/9)	22.2 (2/9)	55.6 (5/9)	66.7 (12/18)	11.1 (2/18)	0 (0/18)

[a] (Number of on-target mutant genotype/total number of on-target mutant genotypes) x 100%.
[b] (Number of allele mutation type/number of all allele mutation types) x 100%.

282

283 ## Inheritance of CRISPR/Cas9-induced mutations in the T_1
284 ## generation

285 To assess the inheritance of the CRISPR/Cas9-induced *OsSWEET14* mutations in the
286 next generation, all T_0 mutant transgenic plants (Fig 1C) were allowed to self-pollinate,
287 and T_1 transgenic plants were randomly selected in the progeny of T_0 plants for
288 sequencing and analysis of their edited site (Table 2). All T_1 individuals derived from T_0
289 plants previously genotyped as homozygous possessed the same allele as their parent,
290 suggesting stable inheritance of the mutations to the next generation. Similarly, the T1
291 progeny of each of both bi-allelic and heterozygous mutation T_0 lines showed a
292 segregation ratio which is consistent with Mendelian segregation ($\chi^2 < \chi^2_{0.05,\ 2} = 5.99$),
293 indicating that the CRISPR/Cas9-induced mutations in T_0 plants were transmitted as
294 expected to the next generation. Interestingly, no new mutant allele was detected in the T_1
295 generation of both heterozygous mutants L-21 and L-27, even though most of them still
296 carried the transgene. Overall, consistent with previous similar studies, our results
297 indicate that the CRISPR/Cas9-mediated mutations generated here are stably transmitted
298 to the next generation in a Medelian fashion.

299 **Table 2. Transmission of CRISPR/Cas9 editing events to the T_1 generation.**

T_0 plant	**Genotype**	**Allele(s)**	**No. of T_1 plants tested**	**Mutation inheritance in the T_1 generation**		**No. of T-DNA-free plants**
				Alleles segregation	**χ^2 (1:2:1)**	

L-4	Bi-allelic	-5/-3	32	10 (-5), 18 (-5/-3), 4 (-3)	2,750	5 (2*)
L-5	Bi-allelic	-6/+1	44	9 (-6), 22 (-6/+1), 13 (+1)	0,727	10 (2*)
L-7	Bi-allelic	-4/-3	38	14 (-4), 17 (-4/-3), 7 (-3)	3,000	11 (4*)
L-15	Homozygote	+1	5	5 (+1)	-	1 (1*)
L-21	Heterozygote	-3	26	3 (-3), 13 (-3/wt), 10 (wt)	3,769	7 (1*)
L-27	Bi-allelic	-5/-4	7	1 (-5), 3(-5/-4), 3 (-4)	1,286	0
L-29	Heterozygote	-5	33	6 (-5), 19 (-5/wt), 8 (wt)	1,000	2 (0*)
L-31	Homozygote	-3	15	15 (-3)	-	5 (5*)
L-54	Bi-allelic	-3/-2	21	3 (-3), 12 (-3/-2), 6(-2)	1,286	3 (0*)

"+" and "-" indicate respectively, insertion and deletion, of the indicated number of nucleotides.
"w", wild type.
*Number of homozygous mutant plants without T-DNA.

Selection of transgene-free mutant TBR225 rice lines

To identify T-DNA free T_1 rice plants containing a mutation in EBEs of the *OsSWEET14* promoter, PCR analysis was carried out using primers specific to *Cas9*, sgRNA and *HPT* sequences (Table 2). A T1 individual was considered devoid of the transgene if the control amplification of the *OsSWEET14* promoter was successful and if none of the PCR reactions with independent primer pairs designed on the T-DNA produced a detectable diagnostic band. The results of this PCR screen show that the T-DNA could be segregated out in the progeny of most T_0 lines, with 88.9% of the T_0 lines generating T-DNA-free progeny. In total, 44 of 221 analyzed edited T_1 plants did not generate a specific amplicon from the T-DNA construct and 15 of them were homozygous mutant

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311 harboring the desired *OsSWEET14* modifications. Our results demonstrate that transgene-
312 free, homozygous mutant individuals could be obtained in the segregating progeny of
313 selfed T_0 individuals.

314

315 TBR225 *OsSWEET14* promoter editing confers resistance to 316 Vietnamese *Xoo*

317 To characterize the BLB-resistance phenotype of the generated rice mutants, three T-
318 DNA-free, homozygous TBR225 edited lines, namely, L-5.7(-6), L-31.12(-3) and L-
319 15.4(+1) with *OsSWEET14* promoter alleles corresponding respectively to L-5-a1 (6bp
320 deletion), L-31 (3bp deletion) and L-15 (1bp insertion) in Fig 1C, were established.
321 Selected T_1 individuals were propagated to obtain T_2 seeds which were used to perform
322 BLB susceptibility assays. Edited T_2 and WT TBR225 plants were inoculated by leaf-
323 clipping with the VXO_11 and VXO_15 strains at the eight-week stage. The inoculated
324 leaves of wild type TBR225 plants and of edited lines L-15.4(+1) and L-31.12(-3)
325 developed long water-soaked lesions typical of BLB, ranging from 18.3 cm to 29.0 cm in
326 length. In contrast, the edited line L-5.7(-6), harboring a longer 6-bp deletion at the target
327 site, displayed high (1.2 cm average lesion length) and moderate (7.3 cm average lesion
328 length) resistance to VXO_11 and VXO_15 strains, respectively (Fig 3). Means
329 comparisons with a Tukey's HSD test further indicated that irrespective of the inoculated
330 strain, the mean lesion lengths measured on the L-15.4(+1), L-31.12(-3) or wild type

331 lines were not significantly different. In contrast, the mean lesion lengths recorded on the
332 L-5.7(-6) mutant line were significantly different from those obtained on the wild type
333 and the two other edited lines challenged with either of the Vietnamese strains (Fig 3B).
334 Furthermore, our off-target editing analysis on line L-5.7(-6) did not reveal unintended
335 modifications of other annotated rice loci (Table S2 and Figure S5), indicating that the 6-
336 bp deletion in the *OsSWEET14* promoter is probably responsible for this phenotype.

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337 Consistent with disease assays and as shown in Figure 3C, whereas a semiquantitative
338 RT-PCR signal for *OsSWEET14* expression was detected on the parental variety and the
339 L-15.4(+1) and L-31.12(-3) edited lines following VXO_11 and VXO_15 infiltration,
340 this amplicon was undetectable in the resistant L-5.7(-6) line.

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341

342 In conclusion, this data shows that ~~whereas~~ the ~~a~~ 6-bp deletion in the AvrXa7/PthXo3
343 EBE reduces dramatically *OsSWEET14* expression following VXO strains inoculation
344 and confers ~~measureable~~ resistance to ~~VXO~~these strains. In contrast, shorter
345 modifications on the 3'-end of this EBE are insufficient to perturb *OsSWEET14*
346 expression after inoculation and do not confer detectable protection ~~to~~ against the
347 corresponding strains. ~~Furthermore~~Finally, while these results strongly support the view
348 that *OsSWEET14* functions as a unique susceptibility gene in the interaction between
349 strain VXO_11 and the TBR225 rice variety, the resistance to strain VXO_15 is not as
350 dramatic and may suggest that other mechanisms partially counteract the effects of the
351 AvrXa7/PthXo3 EBE 6-bp deletion in edited TBR225 plants.

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352

353 **Fig 3. BLB resistance assays for homozygous mutant rice lines L-5.7(-6), L-15.4(+1)**

354 **and L-31.12(-3)**. (A) Leaves were photographed 14 days post-leaf clipping inoculation of

355 *Xoo* strains VXO_11 and VXO_15; arrow heads indicate the end of the lesion. (B) Mean

356 lesion lengths (bars) and standard deviations (error bars). Values were measured 14 days

357 post-leaf clipping inoculation of two *Xoo* strains VXO_11 and VXO_15 and were

358 computed from at least three leaves from each of three plants. Asterisks indicate

359 significant differences relative to wild type plants (Tukey's HSD test; ** $P < 0.05$). The

360 number in the parentheses following the line name indicates the type of mutation and the

361 number of nucleotides involved. The letters above strain labels indicate susceptibility

362 score (R - high resistance; M – moderate resistance; S - susceptibility). The experiment

363 was repeated three times. (C) *OsSWEET14* expression pattern obtained by RT-PCR two

364 day post-infiltration of genome edited homozygous mutant rice lines L-31.12(-3), L-

365 15.4(+1) and L-5.7(-6) and parental TBR225 rice leaves with Vietnamese *Xoo* strains.

366 This experiment was repeated two times with similar results.

367

368

369 **TBR225 *OsSWEET14* promoter edited lines agronomic**

370 **performances are undistinguishable from the parental variety**

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371 To determine if ~~and how~~ mutations in the *OsSWEET14* promoter affect agronomic traits
372 of TRB225 rice plants, three independent homozygous mutant lines were analyzed by
373 measuring their growth duration, plant height, number of tillers per plant, number of
374 grains per panicle, number of filled grains per panicle, yield per plant and amylose
375 content under net-house conditions (see picture of S3 Fig). ANOVA tests and Student's *t*
376 tests showed that the mutant lines displayed no significant difference to TBR225, in
377 terms of the examined agronomic traits, under our net-house conditions (Table 3). These
378 results suggest that the tested CRISPR/Cas9-induced mutations in the *OsSWEET14*
379 promoter did not negatively impact the main agronomic traits of TBR225.

380

381 **Table 3. Agronomic traits evaluation of homozygous T_2 mutant lines.**

Lines	**Growth duration (day)**	**Plant height (cm)**	**No. of tillers per plant**	**No. of grains per panicle**	**No. of filled grains per panicle**	**Amylose content (%)**
WT	108.4 ± 1.1[a]	86.6 ± 3.2[a]	5 ± 0.7[a]	144.4 ± 4.9[a]	125 ± 4.5[a]	13.2 ± 0.38[a]
L-5.7(-6)	108 ± 1.2[a]	86.4 ± 4.3[a]	5.2 ± 0.4[a]	144.2 ± 4.4[a]	123.4 ± 5.5[a]	13.7 ± 0.35[a]
L-15.4(+1)	107.8 ± 0.8[a]	86.4 ± 5.0[a]	4.8 ± 0.4[a]	147.8 ± 5.1[a]	121.8 ± 3.0[a]	13.5 ± 0.41[a]
L-31.12(-3)	108 ± 1.2[a]	88.4 ± 4.3[a]	5.4 ± 0.5[a]	144.6 ± 5.3[a]	124.2 ± 7.4[a]	13.8 ± 0.21[a]

Five plants per line were measured. Experiments were repeated three time.
Means followed by the same letter do not differ significantly ($P < 0.05$).

382

383

384 ## Discussion

385 Recently, the CRISPR/Cas9 system has emerged as a powerful tool for gene editing in
386 many organisms including plants. Because of its specificity and efficiency, this system

387 has been widely used to improve important agronomic traits of major crops such as rape,
388 tomato, soybean, rice, wheat and maize [32]. Excluding easy-to-transform reference
389 accessions such as Nipponbare and Kitaake that are widely used in the laboratory, the
390 number of reports on the improvement of agriculturally relevant elite rice cultivars for
391 pertinent traits using the CRISPR/Cas9 technology (see for example [33–36]) is gradually
392 increasing but is still limited.

393 TBR225 [24], a major commercial rice variety cultivated in large areas of Northern
394 Vietnam, has the advantages of early maturity, high and stable yield, as well as cooking
395 quality. However, it is very susceptible to BLB. Here, the CRISPR/Cas9-mediated
396 editing method was applied in order to rapidly improve the BLB resistance of TBR225 by
397 modifying the AvrXa7, PthXo3 and TalF EBEs on the promoter of *OsSWEET14*. Of the
398 three generated homozygous mutant lines tested for resistance, the one carrying the
399 largest deletion at the target site (6 bp) showed a significantly improved resistance to
400 infection with two *Xoo* strains VXO_11 and VXO_15. Therefore, using the major
401 commercial rice variety TBR225 as an example, we illustrate the advantages of
402 CRISPR/Cas9 tool for rice breeding.

403 In the present study, the frequency of individuals with CRISPR/Cas9-induced mutations
404 in T_0 transgenic plants was 90%, which is similar to previous observation [28]. We
405 obtained only two heterozygous mutant/wild type lines versus seven homozygous or bi-
406 allelic mutant lines. This high frequency of mutated alleles is another proof that the
407 CRISPR/Cas9 system is indeed an efficient tool for gene editing in plant. We also

408 observed the stable transmission of edited alleles to subsequent generations. This is a
409 common phenomenon that has been repeatedly documented for rice plants carrying
410 CRISPR/Cas9-induced mutations [33,35]. In this study, we obtained only two types of
411 induced mutations ~~were observed~~ in T_0 plants: ~~single-nucleotide~~ insertion (11.1%) and
412 deletion (66.7%), but no substitution ~~or combinations of the different mutation types~~were
413 observed. In some earlier studies, new mutations were continuously obtained in the T_1
414 offspring of heterozygous T_0 mutants because ~~of the continuous activity of~~ the
415 ~~CRISPR/~~Cas9 complex remains active on edited targets until the seed or PAM
416 regions~~system~~ cease to be functional [35,37,38]. In contrast, here, all the T_1 plants
417 generated from both heterozygous lines L-21 and L-29, regardless of whether they had a
418 CRISPR/Cas9 T-DNA transgene integrated in their genome, did not show any new
419 mutation possibly because CRISPR/Cas9 T-DNA transgene was no longer functional.
420 We could also readily obtain transgene-free plants from most of the T_1 segregation
421 populations without any laborious crossing or backcrossing steps, which illustrates an
422 advantage of the CRISPR/Cas9 technology compared to conventional breeding.

423 Clade III SWEET family proteins are involved in a number of biological processes such
424 as seed and pollen development or pathogen susceptibility [39]. Their inactivation has
425 previously been shown to cause pleiotropic and/or detrimental effects. For example, both
426 *ossweet11* single and *ossweet11-ossweet15* double Kitaake rice mutants showed defects
427 in endosperm development and filling [40]. In addition, RNA-mediated silencing of
428 either *Os11N3/OsSWEET14* [7] or *Os8N3/OsSWEET11* [6] in BLB resistant Kitaake

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lines causes negative effects on seed production. In contrast, here, we show that T-DNA-free TBR225 plants harboring homozygous mutations generated with the CRISPR/Cas9 system in the AvrXa7/PthXo3 EBE of the *OsSWEET14* promoter exhibited enhanced *Xoo* resistance but did not show any significant difference in all examined agronomic traits compared to wild-type plants under net-house growth conditions. It is conceivable that limited modifications in promoter regions do not affect the normal expression of *SWEET* genes in contrast to KO or silenced lines. Our findings are consistent with the previous work of Oliva et al. [11] who studied 30 combinations of EBE mutations in the *OsSWEET11*, *OsSWEET13* and *OsSWEET14* promoters of the IR64 or Ciherang-Sub1 varieties and detected only a single line with abnormal agronomic traits.

Some individual *Xoo* strains have evolved a set of distinct TALE effectors that collectively target several members of the clade III SWEET family. The presence of these redundant TALEs thereby trumps single "~~loss~~ loss-~~of~~ of-~~tale~~ tale-responsiveness" resistance alleles [11,12,17,41]. For example, Kitaake lines carrying TALEN-induced mutation in the *SWEET14* promoter [13,15] exhibit resistance to strains which depend exclusively on matching AvrXa7/PthXo3 for clade III *SWEET* family induction. Likewise, the natural *xa13* allele [42] or CRISPR/Cas9-induced mutation in the *SWEET11* promoter [11] exhibit resistance to strains such as PXO99 which depend exclusively on PthXo1, for virulence. However, the BLB resistance of the Kitaake lines harboring mutations in both AvrXa7/PthXo3 (*OsSWEET14*) and PthXo1 (*OsSWEET11*) EBEs ~~were~~ was defeated by *Xoo* strains expressing simultaneously the AvrXa7/PthXo3

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and PthXo2B TALEs [11]. Recently, the stacking of EBE-edited alleles in several *OsSWEET* promoters have overcome this limitation and was shown to achieve a broad spectrum of resistance to strains from most BLB-prone countries in Asia [11,12].

All of the three T_2 lines tested for BLB resistance were affected ~~only~~ for the AvrXa7/PthXo3 EBE and conserved an otherwise wild type TalF EBE (Fig 1C). The homozygous mutant TBR225 line L-5.7(-6) carrying a 6-bp deletion in the AvrXa7/PthXo3 EBE exhibited a significantly enhanced resistance to two Vietnamese *Xoo* strains compared to WT TBR225. The L-15.4(+1) and L-31.12(-3) lines that harbored more subtle alterations in the 3'-end of this EBE (a 1-bp insertion and a 3-bp deletion, respectively) in contrast remained susceptible to VXO strains. Our *OsSWEET14* expression analysis after Vietnamese Xoo strains inoculation (Fig 1C) suggests ~~It is therefore possible~~ that these editing events did not alter the EBE sequence sufficiently to compromise promoter recognition by an AvrXa7/PthXo3-like Vietnamese TALE. With less than 2 cm average lesion length, the resistance of line L-5.7(-6) (6-bp deletion) to the VXO_11 strain is rather extreme (versus average lesion length of 20.1 cm on wild type plants). Moreover, in this line, *OsSWEET14* expression following bacterial inoculation is strongly reduced relative the parental line and the two other edited lines, which suggest that in this case, recognition by an AvrXa7/PthXo3-like Vietnamese TALE is abrogated. Consistent with ~~this interpretation~~ *OsSWEET14* expression analysis and as shown in S4 Fig, the Talvez [43] target prediction scores for AvrXa7 and PthXo3 on the *OsSWEET14* promoter L-5-a1 allele sequence of line L-5.7(-6) are markedly lower than on the wild

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type promoter sequence. This is not the case however for the edited alleles carried by lines L-15.4(+1) and L-31.12(-3) (respectively L-15 and L-31 in S4 Fig) whose Talvez scores are identical or slightly lower than those of the wild type promoter sequence.

The magnitude of the effect of the 6-bp deletion allele on susceptibility to VXO_11 is comparable to the dramatic effect of previously characterized alterations of the same EBEs in the Kitaake background against the PXO86 strain that possesses a single TALE, AvrXa7, targeting *OsSWEET14* for clade III *OsSWEET* gene induction [15]. By analogy, this suggests that *OsSWEET14* is also the only clade III *OsSWEETs* target of VXO_11 in the TBR225 background but, in order to confirm this hypothesis an examination of other clade III *OsSWEET*~~s~~ genes expression ~~induction~~ patterns in response to this strain would be required. The situation with the VXO_15 strain is not as straightforward to interpret and will require further investigations. Although the 6-bp deletion in the AvrXa7/PthXo3 EBE did provide an increased resistance to the edited plants, the VXO_15 strain caused intermediate disease severity ~~was still intermediate against the VXO_15 strain~~ (7.3 cm average lesion length on Fig 3). This incomplete resistance is unlikely to ~~could~~ result from the partial but still productive recognition of subsequences of the altered EBE by a VXO_15 AvrXa7/PthXo3-like TALE because *OsSWEET14* expression is similarly decreased in response to either this strain or VXO_11 (Fig 3C). Alternatively, ~~analogous to other Asian strains, VXO_15 may encode additional alternative TALEs, such as PthXo2B or PthXo1 that compensate the putative loss of *OsSWEET14* induction by targeting other clade III *OsSWEET* genes. C~~contrary to all Asian *Xoo* examined so far,

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~~(and thus less likely)~~ but similar to African *Xoo* [15], VXO_15 may have the intrinsic potential to cause disease in the absence of clade III *OsSWEET* gene induction. More likely, analogous to other Asian strains, VXO_15 may encode alternative TALEs, such as PthXo2B or PthXo1 that compensate the loss of *OsSWEET14* induction by targeting other clade III *OsSWEET* genes. In this regard, long read genome sequencing will ultimately help describe ~~These results nonetheless suggest that there are at least two groups within Vietnamese *Xoo* in terms of~~ TALEs ~~diversity~~variability in Vietnamese *Xoo* strains.

In conclusion, we showed that editing specific EBEs of *Xoo* TALEs via CRISPR/Cas9 tool is an efficient method for improving BLB resistance of elite rice varieties such as TBR225 without detectable yield penalties. This also uncovered the potential diversity of TALEs in Vietnamese *Xoo* population, which will thus require future investigations to address the TALE repertoires of Vietnamese *Xoo* strains in order to generate broad-spectrum BLB-resistant rice varieties in Vietnam.

Authors' Contributions

Nguyen Duy Phuong and Tran Lan Dai are equal contributors

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Supporting information

S1_raw_images. Original photograph used in Fig 2 for the RT-PCR gels panel.

S2_raw_images. Original photograph used in Fig 3C for the RT-PCR gels panel.

S1 Fig. Nucleotide sequence of the *OsSEET14* promoter in TBR225.

S2 Fig. Virulence of Vietnamese *Xoo* strains VXO_11 and VXO_15 on TBR225 rice. Grey points correspond to individual lesion length measurements while the black points indicate the calculated average value. The line range represents standard deviation.

S3 Fig. Picture of an individual plant from the homozygous mutant rice lines L-5.7(-6).

S4 Fig. Talvez scoring of AvrXa7, PthXo3 and TalF target EBES in the edited *OsSWEET14* promoter allele sequences. Score values are represented both by the length of the ~~a horizontal~~horizontal -bar and a fill color scale. Higher Talvez prediction scores reflect a better match between a predicted EBE and the sequence of RVD of the query TALE.

~~S5 Fig.~~ S~~6~~5 Fig. ~~DNA~~Amplicon sequencing ~~analysis~~ of predicted off-target sites for the *OsSWEET14* promoter-sgRNA in annotated exons of the TBR225 edited line L-5.7(-6). Potential unintended target sequences including the PAM ~~Off-target sequences~~ are ~~were showed~~highlighted -in ~~the~~ boxes. They are all identical to the expected wild type Nipponbare sequences.

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846 **S1 Table. Key figures on the TBR225 transformation procedure for *OsSWEET14***

847 **promoter editing.**

848 **S2 Table. Output of the CCTop tool used with the *OsSWEET14* promoter sgRNA**

849 **for off-target prediction on the rice Nipponbare genome.Off-target sequences of**

850 ***OsSWEET14*-sgRNA**

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RESPONSE TO REVIEWERS

You will find below the itemized list of our replies to the comments made about our re-submission.

Review Comments to the Author

Reviewer #1

	Reviewer's Comments	Responses
1	Though there are still some discrepancies that could have improved the manuscript, authors have adequately justified those. For example, they assume that 6 base pair deletion in the promoter region can make a difference in TALE binding but not in gene expression or induction of expression. While working on a promoter editing, it is generally expected to check the expression pattern of the gene under normal and infested condition after editing. Since they did not find any difference in phenotype and it was probably difficult to conduct the experiment, they have avoided the experiment. Authors claim in the conclusion that the study uncovered potential diversity of TALEs. Though the discussion made by authors indicate towards it, they have not done any experiment to verify that. Hence the statement must be modified accordingly.	As also described in our response to comment #3 of Reviewer 2, we have performed the experiment recommended by the reviewers and the results have been integrated in this version of the manuscript (Figure 3C and results section). We believe that this new data (loss of *OsSWEET14* expression in response to both VXO_11 and VXO_15) brings more indirect support to our hypothesis that VXO_15 encodes additional TALE(s) capable of targeting other *OsSWEET* genes. We modified the discussion to accommodate this new data and rephrased this section to attenuate the strength of our statement.
2	Though most of the grammatical errors have been rectified still there are some errors as mentioned below: - Ln 33: 'All examined agronomic traits of three transgene-free T2 lines were not significantly different from those of wild-type TBR225' may read as 'None of the examined agronomic traits of three transgene-free T2 lines were significantly different from those of wild-type TBR225' - Ln 67: recessive resistance? – I pointed	- This was revised as suggested by the Reviewer

	- it earlier, but the explanation was more confusing and must be addressed - Ln 381: The single nucleotide mutation was observed only in two of nine plants. So the type of mutation should be only insertion or deletion but not single nucleotide insertion or deletion	- This was revised as "resistance" - This was corrected as suggested by the Reviewer

Reviewer #2

	Reviewer's Comments	Responses
1	1. The reply to my comment 3 is not satisfactory scientifically. If authors need to back their gene selection in a rational way, they should cite earlier paper that described Asian strains target *SWEET14* or *SWEET13*. For easy reference see below my comment and author's response- My original comment 3: I am wondering if Vietnamese Xoo VXO_11 and VXO_15 strains are known to secrete AvrXa7/PthXo3 from any earlier studies. If not, then how the authors have selected *SWEET14* for expression analysis and then editing the EBEs? How the authors hypothesized that *SWEET14* is the probable target *S* gene for *Xoo* VXO_11 and VXO_15 strains? Authors replied: As thoughtfully pointed out by the reviewer below, based on previous studies, we knew that Asian strains tend to target either *OsSWEET14* or *OsSWEET13*. So, we just tested OsSWEET14 induction and were very lucky it turned to be the good choice.	We apologize if we do not fully grasp the point of the reviewer. Candidate gene selection could in principle be entirely random and still yield scientifically sound and serendipitous results in the end. Nevertheless, we believe we provide a reasonable rationale for why focus on *OsSWEET14*: - in the introduction, we comment on the specificity of *OsSWEET* gene targeting by Asian strains in the paragraph "Previous studies established that rice resistance to *Xoo* resulting from "TALE-unresponsive" alleles can be conferred by natural DNA polymorphisms […] BLB resistance engineering thus required multiplex *OsSWEET* promoters EBE editing using the CRISPR/Cas9 system [11,12]." and extensively cite the literature that previously addressed this issue. - we begin the result section with this sentence "*OsSWEET14/Os11N3* was previously identified as a susceptibility gene for *Xoo* strains relying on either of the AvrXa7, PthXo3, TalF (formerly Tal5) or TalC TALEs for infection of the rice cultivars Nipponbare and Kitaake". We modified the following sentence to say that because it is often targeted by Xoo strains, we focused our initial work on this promoter.
2	Figure caption: S4: Explain a bit more about TALVEZ scoring for making it easy for the	We added a sentence in the Figure legend briefly explaining the nature of the Talvez score and making it

	readers	clear that it increases with the likeliness of a candidate EBE being a genuine target based on the RVD-nucleotide association code.
3	Similarly, reply to my original comment 6 is not satisfactory. It is not understood why authors are reluctant to perform expression analysis. This experiment needs to be done, otherwise the manuscript looks like a substandard one. Original comment 6: For another line of confirmation, expression analysis of SWEET14 gene from the edited lines (before/after infection) would be a great addition. Authors discussed “This incomplete resistance could result from the partial but still productive recognition of subsequences of the altered EBE by a VXO_15 AvrXa7/PthXo3-like TALE.” This could be simply analysed by expression analysis in the edited line. Authors replied: We agree that examining OsSWEET14 expression in the edited lines would help decide between possible explanations for the partial resistance phenotype against VX0_15. As described in our reply to Reviewer 1's comment #1, we however believe this is beyond the scope of the core results of our study. To tackle this issue, we are in the process of generating the resources to obtain a good vision of the tal genes content of some VXO strains (including VXO_11 and VXO_15). This and the suggested expression assays will be part of a follow up study focusing on the mechanisms explaining these phenotypes	At the reviewers' request, we have performed additional experiments and have included this data in a new panel of Figure 3. The corresponding *OsSWEET14* expression data in the parent and edited lines following Vietnamese strains inoculation is consistent with the interpretation that only the most dramatic 6bp deletion in the AvrXa7 EBE abrogates *OsSWEET14* upregulation and causes resistance to the examined Vietnamese Xoo strains.

4	Authors have not performed off-target analysis even for revised manuscript. Which is a standard practice for performing CRISPR-Cas9 experiment. Authors used a single guide and analyzing off-targets for a single guide is an easy task	At the reviewer's request, we have performed off-target sites predictions with the sequence of the gRNA used for editing and selected three likely potential unintended target sites located in annotated exons for PCR amplicon sequencing with genomic DNA from the resistant L-5.7(-6) line. This analysis did not detect genome modifications and the results were included in the new version of the manuscript (Table S2, Figure S5, results section).
5	Authors have not taken care of the following original comment in their discussion in the revised manuscript. Line number 382-390 in the revised manuscript. Original comment: other 5: Line 347-349: See the discussion of an earlier publication (https://doi.org/10.1007/s42994-020-00018-x). The authors may cite and take help from the paper to discuss additional mutations in T1 generation. In the Page 116 of the suggested paper, it is discussed "Plants descendent from mutants generated by active Cas9 are prone to further rounds of editing until the PAM and seed region of protospacer are destroyed by editing." Please also discuss your result in this line	We have added this reference to the list of papers we previously cited in this section of the discussion and have revised our sentence to convey the notion suggested by the reviewer.