CRISPR/Cas9-targeted mutagenesis of Os8N3 in rice to confer resistance to Xanthomonas oryzae pv. oryzae

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Abstract

Background: Genome editing tools are important for functional genomics research and biotechnology applications. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 (Cas9) system for gene knockout has emerged as the most effective genome-editing tool. It has previously been reported that, in rice plants, knockdown of the Os8N3 gene resulted in enhanced resistance to Xanthomonas oryzae pv. oryzae (Xoo), while displaying abnormal pollen development.

Results: The CRISPR/Cas9 system was employed to knockout rice Os8N3, in order to confer enhanced resistance to Xoo. Analysis of the genotypes and edited Os8N3 in T₀, T₁, T₂, and T₃ transgenic rice plants showed that the mutations were transmitted to subsequent generations, and homozygous mutants displayed significantly enhanced resistance to Xoo. Stable transmission of CRISPR/Cas9-mediated Os8N3 gene editing without the transferred DNA (T-DNA) was confirmed by segregation in the T₁ generation. With respect to many investigated agronomic traits including pollen development, there was no significant difference between homozygous mutants and non-transgenic control plants under greenhouse growth conditions.

Conclusion: Data from this study indicate that the CRISPR/Cas9-mediated Os8N3 edition can be successfully employed for non-transgenic crop improvements.

Keywords: CRISPR/Cas9, Disease resistance, Os8N3, Rice, xa13, Xanthomonas oryzae pv. oryzae

Background

Rice (Oryza sativa L.) is one of the most important cereal crops in the world, directly feeding more people than any other crop. Bacterial blight, caused by Xanthomonas oryzae pv. oryzae (Xoo), is a prevalent and destructive rice disease that causes serious production loss worldwide (Zhang and Wang 2013). Enhancing rice plants’ resistance to Xoo is known to be an economical and effective approach for managing rice bacterial blight.

Xoo pathogenicity depends on a specific class of virulence factors, called transcription activator-like (TAL) effectors, which mimic plant transcriptional activators (Hutin et al. 2015; Blanvillain-Baufume et al. 2017). The TAL effectors target the host nucleus, where they bind to specific promoter elements of the plant genes and activate their expression, reprogramming the plant transcriptome (Schornack et al. 2013). The genomes of Xanthomonas strains typically contain highly variable numbers of TAL effectors between Asian Xoo (15–26), African Xoo (8–10), and North-American Xoo (0) (Erkes et al. 2017). The rice genes targeted by TAL effectors have been identified as host disease-susceptibility genes, acting as major susceptibility factors during rice and Xoo interactions. In some cases, DNA polymorphisms in the so-called TAL effector binding elements (EBEs), located at the promoter region of the susceptibility gene, lead to no development of the disease (Yang et al. 2006; Hutin et al. 2015). Rice Os8N3 (also known as OsSWEET11), which belongs to the Sugar Will Eventually be Exported Transporters (SWEET) family of sugar transporters, represents one of the susceptibility genes induced by TAL effectors (Yang et al. 2006;
The expression of Os8N3 is induced by strains of Xoo carrying pthXo1, which encodes the TAL effector PthXo1 (Yang et al. 2006; Yuan et al. 2009). PthXo1 from Xoo strain PX099 directly activates Os8N3 through recognition of TAL EBEs located at the promoter region of Os8N3 (Romer et al. 2010). The recessive resistance gene xa13 occurs as a series of natural alleles of the susceptibility gene Os8N3 (Yang et al. 2006; Yuan et al. 2009). Although it has not been clearly demonstrated, Os8N3 is believed to remove toxic copper from xylem vessels where Xoo multiplies and spreads (Yuan et al. 2010), and make nutrients easily available to Xoo for its growth and virulence to cause disease (Chen et al. 2010; Chen et al. 2012).

Genome editing technologies enable precise modification of DNA sequences in vivo and promise a novel revolution in crop improvement (Sun et al. 2016; Feng et al. 2013). The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 (Cas9) system has revolutionized genome editing and become widely popular because of its specificity, simplicity, and versatility. It allows targeted genome editing in organisms guided by a customizable small noncoding RNA called single guide RNA (sgRNA). Once susceptibility genes targeted by TAL effectors have been identified, the CRISPR/Cas9-mediated genome editing strategy can be employed to create a target mutation in the susceptibility genes. Although it was not edited by the CRISPR/Cas9, Os11N3 (also known as OsSWEET14), the susceptibility gene targeted by AvrXa7 and PthXo3, has been edited by Transcription Activator-Like Effector Nuclease (TALENs) to create bacterial blight-resistant rice through disrupting the EBE site in the promoter region (Li et al. 2012; Blanvillain-Baufume et al. 2017). It can also be applied to negative regulators of disease resistance that have been studied for the last decades (Grand et al. 2012; Blanvillain-Baufume et al. 2017). Recently, the efficiency of mutations targeted by sgRNAs driven by different small nuclear RNA promoters including OsU3, OsU6a, OsU6b, and OsU6c, were compared in an Indica cultivar 93–11 (Ma et al. 2015b). OsU6a was slightly more efficient in driving genome editing than the other promoters. It has also been reported that U6 promoters derived from the target plants function better than heterologous U6 promoters (Sun et al. 2015). Therefore, it was decided to use the OsU6a promoter isolated from the Japonica cultivar Kitaake. The OsU6a promoter amplified from Kitaake contains five single-nucleotide substitutions and one 5-bp deletion compared with one from Indica cultivar 93–11 (Additional file 1: Figure S1). The Arabidopsis U6 promoter in the CRISPR/Cas9 vector, pHATc (Kim et al. 2016), was replaced with the Kitaake OsU6a promoter, and the resulting OsU6a:pHATc was used for rice CRISPR/Cas9-mediated target mutagenesis.

**Results**

**Os8N3 in the rice cultivar Kitaake**

Os8N3 was originally isolated as a susceptibility gene from the rice cultivar Nipponbare (Yang et al. 2006) and later, the EBE in its promoter element bound and activated by TAL effector PthXo1 of PXO99 was determined experimentally (Romer et al. 2010). In this study, rice cultivar Kitaake was investigated to see if it also carries the EBE sequence in the Os8N3 promoter region. Using the Kitaake database (Li et al. 2017), the promoter sequence of the Os8N3 gene, ranging from −1000 bp to −1 bp relative to the ATG start codon, was analyzed (Fig. 1a). The putative TATA box (TATAAA) is located at −32 upstream of the transcription start site (+1). The promoter region including PthXo1 EBE (TGCATC TCCCCCTACTGTACACC), ranging from −80 bp to −56 bp upstream of the transcription start site, displayed 100% identity to Nipponbare (Yang et al. 2006). After inoculation with strain PXO99, Kitaake displayed strong induction of Os8N3 two days after inoculation (DAI) (Fig. 1b) and long water-soaked lesions (approximately 13–14 cm) 12 DAI (Fig. 1c). These results suggest that Kitaake carries a functional susceptible gene Os8N3, whose expression is induced by PXO99 possessing the TAL effector PthXo1.

**CRISPR/Cas9 design for xa13/Os8N3 editing**

In monocot plants, the rice U3 small nuclear RNA promoter (OsU3) is generally used to express sgRNA (Belhaj et al. 2013). Recently, the efficiency of mutations targeted by sgRNAs driven by different small nuclear RNA promoters including OsU3, OsU6a, OsU6b, and OsU6c, were compared in an Indica cultivar 93–11 (Ma et al. 2015b). OsU6a was slightly more efficient in driving genome editing than the other promoters. It has also been reported that U6 promoters derived from the target plants function better than heterologous U6 promoters (Sun et al. 2015). Therefore, it was decided to use the OsU6a promoter isolated from the Japonica cultivar Kitaake. The OsU6a promoter amplified from Kitaake contains five single-nucleotide substitutions and one 5-bp deletion compared with one from Indica cultivar 93–11 (Additional file 1: Figure S1). The Arabidopsis U6 promoter in the CRISPR/Cas9 vector, pHATc (Kim et al. 2016), was replaced with the Kitaake OsU6a promoter, and the resulting OsU6a:pHATc was used for rice CRISPR/Cas9-mediated target mutagenesis.

To design a CRISPR/Cas9 that targets the Os8N3 gene, a 20-bp nucleotide sequence (xa13m) in the first exon of Os8N3 was chosen as the target site (Fig. 2a). The xa13m
targeting sequence and protospacer adjacent motif (PAM) sequence are represented in red and in underlined lower-case letters, respectively. The predicted Cas9 cleavage site (vertical arrowhead) in the coding region of the gene was 31 bp downstream from the ATG initiation codon. The recombinant binary plasmid, OsU6a::xa13m-sgRNA/pHAtC, carrying xa13m-sgRNA targeting the Os8N3 gene under the control of the OsU6a promoter, was then constructed based on the OsU6a::pHAtC (Fig. 2b).

CRISPR/Cas9-mediated targeted mutagenesis of xa13/Os8N3

After Kitaake was transformed with OsU6a::xa13m-sgRNA/pHAtC using Agrobacterium-mediated transformation, four independent transgenic Kitaake plants (OsU6a xa13m/Kit T0, 1A, 2A, 3A, and 4A) were generated. The putative transgenic plants were subjected to polymerase chain reaction (PCR)-based selection using the Cas9-specific primers, Cas9_RT_F and Cas9_RT-R (Fig. 2b), and all of them generated a Cas9-specific 400-bp amplicon (Fig. 3a). To further investigate CRISPR/Cas9-targeted mutagenesis of Os8N3, the target-containing amplicons obtained from all PCR-positive transgenic plants were directly sequenced and analyzed by decoding via the Degenerate Sequence Decoding method (Liu et al. 2015; Ma et al. 2015a). Rice plants are diploid with two copies of each gene, one copy on each chromosome of a chromosome pair. Therefore, when CRISPR/Cas9 is inserted into the genome and begins to function, one or both copies of the target gene Os8N3 can be cleaved and mutated, generating five possible genotypes in the transgenic plants: homozygote, biallele, heterozygote, chimera, and wild type (WT). In four T0 transgenic plants, there was only one homozygous mutation, 1-bp insertion (+A), in 4A, whereas no target sequence changes could be detected in the other plants (T0 in Table 1 and Additional file 2: Figure S2).

Inheritance of Os8N3 mutations and enhanced resistance to Xoo

To determine if and how the CRISPR/Cas9-targeted mutagenesis of Os8N3 by OsU6a::xa13m-sgRNA/pHAtC was transmitted to the next generation, all OsU6a
xa13m/Kit T₀ transgenic plants were self-pollinated and the targeted Os8N3 of some T₁ transgenic plants was directly sequenced and analyzed (Fig. 3b, Table 1, and Additional file 3: Figure S3). The homozygous mutated T₀ line (4A) produced homozygous mutated T₁ progeny (4A-1, 4A-2, and 4A-3) and did not display additional different mutations. There was no mutation observed in the sequenced T₁ progenies of the WT 1A, and 2A lines. However, new targeted sequence changes were detected in the T₁ progeny of the WT 3A line. Previously, sequencing results indicated a putative WT genotype of the targeted Os8N3 in the T₀ 3A line, whereas three (3A-2, 3A-4, and 3A-6) out of the five sequenced T₁ progenies of the WT 3A line displayed a 1-bp insertion (Table 1): 3A-2 was homozygous; 3A-4 was bi-allelic; and 3A-6 was heterozygous.

To characterize the bacterial blight resistance phenotype of the mutant lines, T₁ lines (progeny of OsU6a xa13m/Kit 3A-2, 4A-1, 4A-2, and 4A-3) and bi-allelic (3A-4) xa13 mutant plants displayed a robust resistance phenotype compared with heterozygous (3A-6) mutant and Kitaake control plants (T₁ in Table 1 and Fig. 4a). The differences were further evaluated by quantification of the lesion lengths and significance analysis using Tukey’s HSD test (Fig. 4b). Homozygous and bi-allelic mutant plants displaying a resistance phenotype showed no significant differences in lesion lengths compared with the XA21 plants. These results indicated that the homozygous and bi-allelic mutant lines were significantly different from Kitaake and heterozygous mutant plants, and that CRISPR/Cas9-mediated mutagenesis in both Os8N3 alleles conferred robust resistance to PXO99.

To further investigate the inheritance of targeted mutations in later generations, the genotypes of several OsU6a xa13m/Kit T₂ plants were analyzed and inoculated with PXO99. New allelic mutation was detected in the T₂ progeny of WT 1A-5. Although all sequenced T₀ and T₁ generations of the 1A line carry WT Os8N3, T₂ progeny (1A-5-6) of the 1A line displayed a heterozygous 1-bp insertion (+T) mutation (Table 1 and Additional file 4: Figure S4). Heterozygous mutated 3A-6 (+T) produced chimera 3A-6-1 with three distinct alleles detected at the target site, displaying additional different mutations (+A). All T₁ plants derived from the homozygous T₀ mutant typical of bacterial blight disease. Homozygous (OsU6a xa13m/Kit 3A-2, 4A-1, 4A-2, and 4A-3) and bi-allelic (3A-4) xa13 mutant plants displayed a robust resistance phenotype compared with heterozygous (3A-6) mutant and Kitaake control plants (T₁ in Table 1 and Fig. 4a). The differences were further evaluated by quantification of the lesion lengths and significance analysis using Tukey’s HSD test (Fig. 4b). Homozygous and bi-allelic mutant plants displaying a resistance phenotype showed no significant differences in lesion lengths compared with the XA21 plants. These results indicated that the homozygous and bi-allelic mutant lines were significantly different from Kitaake and heterozygous mutant plants, and that CRISPR/Cas9-mediated mutagenesis in both Os8N3 alleles conferred robust resistance to PXO99.

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plant (4A) and T2 plants derived from homozygous T1 mutant plants (4A-1 and 4A-3) were homozygous for the same mutations (Table 1). All homozygous mutant lines (4A-1-6, 4A-1-7, 4A-3-3, and 4A-3-5) and chimera (3A-6-1) displayed significantly short lesion lengths (Fig. 5a and b) and low bacterial populations compared with the heterozygous mutant (1A-5 6) and Kitaake plants (Fig. 5c). These results indicate that the mutations in these homozygous mutant lines and enhanced resistance to PXO99 were stably transmitted to the next generation.

Main agronomic traits in xa13 mutants
To determine whether mutations in the Os8N3 gene affect agronomic traits, two independent homozygous mutant lines (T3) were analyzed by measuring their plant height, flag leaf length/width, the number of productive panicles, and panicle length (Table 2, Additional file 5: Figure S5 and Additional file 6: Figure S6). Tukey’s HSD test indicated that the mutant lines displayed no significant difference to Kitaake, in terms of the investigated agronomic traits, under our greenhouse conditions.

Previously, Os8N3 knockout transgenic plants displayed abnormal pollen development (Yang et al. 2006; Chu et al. 2006). To investigate whether Os8N3 knockout mutations affect pollen development, their pollen developments were assessed (Fig. 6). The phenotypical analysis showed that two independent homozygous T3 mutant lines (3A-6-1-4 and 4A-1-7-6) exhibited normal golden yellow anthers (Fig. 6a). In addition, pollen grains from Kitaake and two independent homozygous T3 mutant lines (3A-6-1-1 and 4A-1-7-1) were stained with iodine potassium iodide (I2-KI) (Fig. 6b). Dark-stained pollen grains (black in color) were considered viable and those that were lightly stained (yellow in color) were considered sterile. Homozygous mutants (3A-6-1-1 and 4A-1-7-1) displayed similar pollen viabilities to Kitaake, under our greenhouse conditions (Fig. 6c). The seed-setting rates and grain fillings were further analyzed in the Os8N3 knockout mutant lines (Additional file 7: Figure S7). Although, under greenhouse conditions, the
Table 1 Transmission and segregation of CRISPR/Cas9-mediated target mutagenesis from T$_0$, T$_1$, T$_2$, and T$_3$ of the OsU6a xa13m/Kit transgenic plant. The recovered mutated alleles of the xa13/Os8N3 gene in the OsU6a xa13m/Kit transgenic plant are shown below the Kitaake sequence. Nucleotide sequences at the target sites are in black capital letters and black dashes. PAM motifs are underlined. Red capital letters indicate the inserted nucleotide. The genotype of the mutation is indicated at the right of each sequence. WT indicates the nucleotide sequences identical to the Os8N3 gene in Kitaake plants. “+” indicates the insertion of the indicated number of nucleotides. No transgene: PCR negative for Cas9 gene; Transgenic: PCR positive for Cas9 gene; S: susceptible to PXO99; R: resistant to PXO99; Not available: inoculation data are not available.

| Line | Target sequence | Editing | Transgene | Zygosity | Phenotype |
|------|----------------|--------|------------|----------|-----------|
| T$_0$ | 1A | CTTCATCAAGCTTACGACGTT | WT | No transgenic | WT | S |
|      | 2A | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3A | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 4A | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | Not available |
| T$_1$ | 1A-1 | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 1A-2 | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 1A-3 | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 1B | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 1C | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 2A | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 2B | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 2C | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3A | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3B | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3C | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 4A | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | R |
|      | 4B | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | R |
|      | 4C | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | R |
| T$_2$ | 1A-3 | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 2A | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 2B | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 2C | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3A | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3B | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3C | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 4A | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | R |
|      | 4B | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | R |
|      | 4C | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | R |
| T$_3$ | 1A-3 | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 2A | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 2B | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 2C | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3A | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3B | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 3C | CTTCATCAAGCTTACGTT | WT | Transgenic | WT | S |
|      | 4A | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | R |
|      | 4B | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | R |
|      | 4C | CTTCATCAAGCTTACGTT | +1 | Transgenic | Homozygous | R |
caryopses from two independent homozygous mutants (3A-6-1 and 4A-1-7-1) were slightly wrinkled as they matured (Additional file 7: Figure S7c), no significant alteration in the seed-setting rate was observed between progeny of two homozygous mutants (3A-6-1 and 4A-1-7) and Kitaake plants (Additional file 7: Figure S7a and S7b).

Selection of transgene-free mutant rice lines

To select rice plants harboring the mutation in Os8N3 but without the T-DNA of the OsU6a::xa13m-sgRNA/pHAtC construct, PCR and phenotypic analysis for the OsU6a xa13m/Kit T0, T1, and T2 plants was performed. Thirty-one segregating T1 plants were analyzed and six of them (19.35%) did not generate a Cas9-specific amplicon from the T-DNA (Fig. 3b). Similarly, PCR analysis also failed to detect the T-DNA in 11 out of the 65 segregating T2 plants (16.92%) derived from nine T1 plants (1A-1, 1A-2, 1A-5, 1A-8, 1A-16, 3A-6, 4A-1, 4A-3, and 4A-7) (Fig. 3c). Notably, the 4A-1 plant was a Cas9-free homozygous mutant harboring the desired xa13/Os8N3 modifications (Fig. 3b and Fig. 4, and Additional file 3: Figure S3 and Additional file 4: Figure S4). None of the seven T2 plants derived from the T1 mutant plant 4A-1 generated the Cas9-specific amplicon (Fig. 3c). Two (4A-1-6 and 4A-1-7) out of the seven carried a 1-bp insertion (+A) and displayed significantly enhanced resistance to PXO99 (Fig. 5), which has also been observed in their parent (4A-1) (Fig. 4). The T3 plant (4A-1-7-1) not generating the Cas9-specific amplicon carried the same Os8N3 modification observed in the T2 mutant plant 4A-1-7 (Fig. 3d and Additional file 4: Figure S4 and Additional file 5: Figure S5). These results indicate that T-DNA-free mutant plants carrying the desired gene modifications can be acquired through genetic segregation in T1, T2, and T3 generations.

Discussion

The CRISPR/Cas9 system has been widely used to provide new avenues in crop improvements in rice, tomato, wheat, and maize (Xu et al. 2015; Feng et al. 2013; Wang et al. 2016; Ito et al. 2015; Wang et al. 2014; Zhou et al. 2014). In this study, OsU6a::pHAtC, which replaced the Arabidopsis U6 promoter in the pHAtC vector (Kim et al. 2016) with the OsU6a promoter of Kitaake, was constructed for rice CRISPR/Cas9-mediated target mutagenesis. Using the OsU6a::pHAtC, targeted mutagenesis in the recessive resistance gene, Os8N3, was generated.
mutant plant 3A-6-1. Because 3A-6 was heterozygous, the presence of a chimeric mutation may result from delayed cleavage in the primary embryogenic cell of 3A-6-1. This chimeric mutation by the CRISPR/Cas9 system is likely a common phenomenon and has been reported in many plant species including rice (Xu et al. 2015; Feng et al. 2013; Wang et al. 2016), Arabidopsis (Feng et al. 2014), and tomato (Ito et al. 2015).

Regarding all examined agronomic traits, there was no significant difference between T3 homozygous mutants and Kitaake plants under greenhouse growth conditions. The homozygous mutant plants had a similar height, flag

**Table 2** Analysis of the agronomic traits of T3 mutant lines

| Plant height (cm) | Flag leaf length (cm) | Flag leaf width (mm) | No. of productive panicles | Panicle length (cm) |
|-------------------|-----------------------|----------------------|---------------------------|---------------------|
| Kitaake           | 69.8 ± 4.1\(^a\)      | 27.8 ± 4.9\(^a\)     | 11.7 ± 0.9\(^b\)         | 3.0 ± 0.0\(^0\)     | 11.1 ± 2.1\(^0\) |
| Progeny of 3A-6-1 | 65.6 ± 6.4\(^a\)      | 26.9 ± 4.0\(^4\)     | 12.7 ± 0.5\(^a\)         | 3.0 ± 0.0\(^0\)     | 12.1 ± 1.4\(^0\) |
| Progeny of 4A-1-7 | 65.7 ± 7.9\(^a\)      | 29.1 ± 4.8\(^4\)     | 12.0 ± 0.4\(^a\)         | 2.5 ± 1.1\(^0\)     | 12.0 ± 2.2\(^0\) |

The results shown are from more than three homozygous mutants of each mutant line, and are represented as the mean ± SE. The values marked with the same letter (*) are non-significantly different (P < 0.050, Tukey’s HSD test)
leaf length and width, number of productive panicles, panicle length, and pollen viability to Kitaake plants. It has been previously reported that Os8N3 is expressed at a high level in panicles and anthers during pollen development (Chu et al. 2006; Yang et al. 2006). Consistent with these observations, although detailed molecular mechanisms have not been elucidated, Os8N3-silenced rice plants displayed reduced fertility, and most pollen grains were defective (Chu et al. 2006; Yang et al. 2006). Therefore, Os8N3, conferring disease resistance by expres-sional loss-of-function in rice, has been considered an essential constituent for pollen development. However, in this study, homozygous mutants in both Os8N3 alleles were generated, and the mutations were stably transmitted to later generations, T₃. The homozygous T₃ mutant plants had normal pollen development, and most pollen grains were well preserved, in comparison with ones from Kitaake plants.

Thus far, it has been believed that Os8N3 plays roles in both copper and sugar transport, indicating its complex function in copper/sugar metabolism and signaling (Chen et al. 2010; Chen 2014; Yuan et al. 2010). However, no one dissected the molecular connection between Xoo resistance by copper/sugar metabolism and pollen development. Among the different in vivo functions of xa13/Os8N3, knockout mutation, in particular, displayed enhanced resistance against Xoo without affecting pollen development. It is not yet understood why OsU6a xa13m/Kit mutant lines did not display the sterile phenotype previously observed in Os8N3-knockdown rice plants (Chu et al. 2006; Yang et al. 2006). Because frameshift mutations of Os8N3 in OsU6a xa13m/Kit lines are located at the very beginning of the Os8N3 polypeptide, it is very unlikely that the mutated polypeptide is functional. Lack of a functional Os8N3 protein in the mutant lines was also supported by a robust resistant phenotype of the homozygous mutant lines, but not heterozygous or Kitaake plants. Therefore, it is possible that there is a novel gene genetically compensating essential pollen development directly or indirectly in homozygous OsU6a xa13m/Kit mutant lines. Genetic compensation was recently proposed to explain increasing numbers of studies revealing phenotypic differences between knockouts and knockdowns in plants (Gao et al. 2015; Braun et al. 2008; Chen et al. 2014) and animals (Young et al. 2009; De Souza et al. 2006; Daude et al. 2012; McJunkin et al. 2011; Law and Sargent 2014; Evers et al. 2016; Karakas et al. 2007; Morgens et al. 2016; Kok et al. 2015; Rossi et al. 2015). For example, similar to Os8N3, there have been studies on Arabidopsis auxin-binding protein 1 (ABP1) that revealed phenotypic differences between knockouts and knockdowns (Gao et al. 2015; Braun et al. 2008; Chen et al. 2014). Inducible abp1 knockdown lines showed defects in shoot and root growth, cell remodeling, or clathrin-mediated endocytosis of PIN auxin efflux carriers (Braun et al. 2008; Paque et al. 2014; Robert et al. 2010). However, abp1 knockout mutants generated by CRISPR/Cas9 are indistinguishable from wild type plants at every developmental stage.

![Fig. 6](image-url) Pollen viability of the homozygous xa13 mutants. a Anthers in mature spikelets of Kitaake, homozygous mutant (T₃, 3A-6-1-4), and homozygous mutant (T₃, 4A-1-7-6). Scale bars, 1 mm. b Representative images of pollen viability tests from Kitaake and homozygous mutants (T₃, 3A-6-1-1 and 4A-1-7-1). Viable pollen grains are stained dark (gray arrow) and sterile pollen grains are stained light yellow (white arrow). Scale bars, 100 μm. c Statistical analysis of pollen viability of Kitaake, homozygous mutants (T₃, 3A-6-1-1 and 4A-1-7-1) lines. Pollen viability percentage was calculated relative to the total pollen counted in three microscopic images.
analyzed (Gao et al. 2015). Although one possible explanation for the difference is off-target effects of ABP1 antisense RNA, it is not yet understood how independent abp1 knockdown lines, which generate fundamentally different approaches for functional down-regulation of the ABP1 gene, display indistinguishable morphological defect phenotypes (Michalko et al. 2016). Recently, genetic compensation was studied in depth on zebrafish (Rossi et al. 2015). While knockdown of zebrafish EGF-like domain 7 (egfl7), an endothelial extracellular matrix gene, leads to severe vascular defects, most egfl7 mutants display no obvious defects (Rossi et al. 2015). Elastin microfibril interactor (Emilin) genes were proposed as compensating genes in the egfl7 knockout mutants (Rossi et al. 2015). Supporting this hypothesis, Os8N3 mutants showed increased expressions of some SWEET genes such as OsSWEET3a, OsSWEET6b, OsSWEET13, and OsSWEET15 (Ma et al. 2017; Yang et al. 2018) and double mutants of Os8N3 and OsSWEET15 displayed much more wrinkled grain morphology, compared with single Os8N3 mutant (Yang et al. 2018). These reports suggest that some of SWEET genes are able to at least partially compensate for the lack of Os8N3. Currently, we are trying to identify candidate genes that compensate for xa13/Os8N3 in the pollen development pathway without affecting Xoo resistance in homozygous mutant lines.

Conclusions
In summary, the CRISPR/Cas9 system was highly efficient in generating Os8N3 gene editing in rice. Mutant lines harboring the desired modification in Os8N3 but without the T-DNA of the OsU6a::xa13m-sgRNA/pHAtC were obtained. T-DNA-free homozygous mutant lines displayed significantly enhanced resistance to Xoo and normal pollen development. This study provides a successful example of improving bacterial blast resistance using CRISPR/Cas9 technology.

Materials and methods
Plant and pathogen materials
Rice cultivar Kitaake (Oryza sativa L. ssp. Japonica) was generously provided by Prof. Pamela Ronald (University of California Davis, USA). Rice plants in this study were maintained in the greenhouse facility at Sejong University in Korea. Xoo strain PXO99 was used in this study. PXO99 was cultured in peptone sucrose agar media (PSA: peptone 10.0 g/L, sucrose 1.0 g/L, L-glutamic acid 1.0 g/L, and agar 16.0 g/L) containing 15.0 mg/L cephalaxin at 28 °C for two days (Bai et al. 2000).

Vector construction
The Gateway™ destination vector, pHAtC binary vector (Kim et al. 2016), was used to construct OsU6a::pHAtC carrying the OsU6a promoter to express sgRNA. A 472-bp DNA fragment containing the OsU6a promoter (Ma et al. 2015c) was amplified from the genomic DNA of Kitaake using primers, EcoRI_OsU6a_F (5′-GGAAATTCCTTTTTTC CTG TAG TT TCCCAC-3′) and XhoI_OsU6a_R (5′- GCTCGAGACACCTGCTCCAAATCCGCGGCAAG CCAGCACCC-3′). The PCR product was cloned into the pGEM®-T Easy Vector according to the manufacturer’s instructions (Promega, USA), and the insert was confirmed by Sanger sequencing. The OsU6a promoter was cut out from the pGEM®-T Easy Vector using EcoRI + XhoI and cloned into the pHAtC, generating an OsU6a::pHAtC vector.

Cloning of sgRNA expression vector
The OsU6a::xa13m-sgRNA/pHAtC vector expressing sgRNA for xa13/Os8N3 (xa13m-sgRNA) was constructed according to the method previously described (Kim et al. 2016). Briefly, the target sequence (xa13m) for Os8N3 editing of Kitaake was designed by the CRISPR-RGEN Tools website (http://rgenome.ibs.re.kr) (Park et al. 2015). The sgRNA templates (xa13m) for Os8N3 were annealed using two primers, 5′-GATTGCTTTGTCATGGCTAACC CGG-3′ and 5′-AAACCCGGTTAGGCATGCAACAGGC -3′, and cloned into AarI-digested OsU6a::pHAtC. Construction of the sgRNA expression vector, OsU6a::xa13m-sgRNA/pHAtC, and its flanking sequences were confirmed by Sanger sequencing.

Rice transformations
Rice transformations were cloned out as previously described (Chern et al. 2005). Agrobacterium tumefaciens strain LBA4404 was used to infect callus tissue induced from Kitaake seeds. Transformants carrying OsU6a::xa13m-sgRNA/pHAtC constructs were selected using hygromycin. Transgenic Kitaake plants overexpressing xa13m-sgRNA (OsU6a xa13m/Kit) were confirmed by PCR using Cas9-specific primers, Cas9_RT_F (5′-CGAGCT GACCAAGGTAGAATGTAACGTACG-3′) and Cas9_RT_R (5′-CGTTGTA TAAGCTTGCAGCCTC-3′).

Expression
For reverse transcription polymerase chain reaction (RT-PCR) analysis of Cas9 and sgxa13 transgenes, total RNA was extracted from fully expanded leaves of OsU6a xa13m/Kit plants using TRizol reagent (Invitrogen, USA). First-strand cDNA was synthesized using quantified RNA (5 μg of total RNA). Expression of Cas9 was confirmed by RT-PCR using Cas9_RT_F and Cas9_RT_R. Meanwhile, the rEFla cDNA fragment was amplified as a control using specific primers, rEFla1048F (5′-ACTGCGCACCATCCTCC CACATTG-3′) and rEFla1552R (5′-CAACAGTCGAAG GGCAATAATAGTC-3′).
Identification of mutant transgenic plants

Rice genomic DNA was extracted from Kitaake leaves and transgenic OsU6a xa13m/Kit plants. All transgenic hygromycin-resistant T₀ plants were analyzed by PCR using the Cas9-specific primers, Cas9_RT_F and Cas9_RT_R. Subsequently, the DNA fragment across the xa13 target site was amplified from the genomic DNAs of all PCR-positive plants using xa13-specific primers, xa13_cas9 60-79_F (5’-CTAGGG-3’) and xa13_cas9 nuclease_R (5’-TGCATGAGCTGAAGCTAGTGCTAGTTTCTAGCTGG-3’). The PCR amplicons were then directly sequenced using primer xa13_cas9 60-79_F. The sequencing chromatograms with superimposed peaks of bi-allelic and heterozygous mutations were decoded using the Degenerate Sequence Decoding method (http://skl.scau.edu.cn/dsdecode/) (Liu et al. 2015; Ma et al. 2015a).

Xoo inoculation and determination of bacterial populations

For Xoo inoculation, Kitaake, XA21, and transgenic OsU6a xa13m/Kit plants were grown in a greenhouse normally until they reached the eight-week stage, unless otherwise stated. PXO99 was used to inoculate rice plants using the scissors dip method (Song et al. 1995; Chern et al. 2005). For lesion length measurements, at least three inoculated leaves were measured to calculate the average and standard deviation 12 days after inoculation (DAI). Representative leaves were photographed 12 DAI. For Xoo colony counts from inoculated leaves 0 and 12 DAI, 20 cm of leaf tissue from the top, including lesions and tissue showing no lesions, was ground up and resuspended in 10 ml water to harvest bacteria. The extract was diluted accordingly and plated out on PSA plates containing 15.0 mg/L cephalaxin. Plates were incubated at 28 °C for two days, and then colony forming units (CFU) were counted. Statistical analysis was performed using Tukey’s HSD test.

Pollen viability tests

Pollen viability was evaluated as previously described (Chhun et al. 2007). Before flowering, six anthers from Kitaake and transgenic OsU6a xa13m/Kit plants were removed and crushed into a fine powder. Pollen grains were stained with 10 μL I₂–KI solution (1% I₂, 3% KI) and 1 μl of stained pollen grains was harvested to observe fertile and infertile pollen under a light microscope. Dark-stained pollen grains were considered viable and the percentage of pollen viability was calculated relative to the total pollen counted in five microscopic images. Seed viability represents the percentage of spikelets that set seed per total number. Statistical analysis was performed using Tukey’s HSD test.

Additional files

Additional file 1: Figure S1. Sequence comparison of OsU6a promoters from Japonica cultivar Kitaake and indica cultivar 93-11. (PDF 72 kb)

Additional file 2: Figure S2. Sequencing chromatogram at the target site of Os8N3 in the CRISPR/Cas9-induced plants (OsU6a xa13m/Kit T₀). The vertical arrowhead indicates an expected cleavage site. (PDF 105 kb)

Additional file 3: Figure S3. Sequencing chromatogram at the target site of Os8N3 in the CRISPR/Cas9-induced plants (OsU6a xa13m/Kit T₁). The vertical arrowhead indicates an expected cleavage site. (PDF 232 kb)

Additional file 4: Figure S4. Sequencing chromatogram at the target site of Os8N3 in the CRISPR/Cas9-induced plants (OsU6a xa13m/Kit T₂). The vertical arrowhead indicates an expected cleavage site. (PDF 196 kb)

Additional file 5: Figure S5. Sequencing chromatogram at the target site of Os8N3 in the CRISPR/Cas9-induced plants (OsU6a xa13m/Kit T₃). The vertical arrowhead indicates an expected cleavage site. (PDF 72 kb)

Additional file 6: Figure S6. Gross morphology of Kitaake and two homozygous Os8N3 mutant lines, T₁ progeny of 3A-6-1 and 4A-1-7. (PDF 72 kb)

Additional file 7: Figure S7. Seed-setting rates of homozygous xa13 mutants. a Representative panicles from Kitaake, homozygous mutant (T₀ 3A-6-1-2), and homozygous mutant (T₀ 4A-1-7-4). b Seed-setting rates of Kitaake, homozygous mutant (progeny of 3A-6-1-1), and homozygous mutant (progeny of 4A-1-7-1). c Mature caryopses of Kitaake, homozygous mutant (T₀ 3A-6-1-2), and homozygous mutant (T₀ 4A-1-7-4). Scale bars, 2.5 mm. (PDF 72 kb)

Abbreviations

CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/CAS9-associated protein-9 nuclease; EBE: Effector binding element; TAL effector: Transcription activator-like effector; Xoo: Xanthomonas oryzae pv. oryzae Philippine Race 6

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Authors’ contributions

YAK, HM and CJP conceived and designed the experiments. YAK and HM performed the experiments and analyzed the data. YAK, HM, and CJP wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

The manuscript has been approved by all authors.

Competing interests

The authors declare that they have no competing interests.

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