Novel alleles of rice elf4G generated by CRISPR/Cas9-targeted mutagenesis confer resistance to Rice tungro spherical virus

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Abstract
Rice tungro disease (RTD) is a serious constraint in rice production across tropical Asia. RTD is caused by the interaction between Rice tungro spherical virus (RTSV) and Rice tungro bacilliform virus. RTSV resistance found in traditional cultivars has contributed to a reduction in the incidence of RTD in the field. Natural RTSV resistance is a recessive trait controlled by the translation initiation factor 4 gamma gene (elf4G). The $Y^{1059V,1060V,1061V}$ residues of elf4G are known to be associated with the reactions to RTSV. To develop new sources of resistance to RTD, mutations in elf4G were generated using the CRISPR/Cas9 system in the RTSV-susceptible variety IR64, widely grown across tropical Asia. The mutation rates ranged from 36.0% to 86.6%, depending on the target site, and the mutations were successfully transmitted to the next generations. Among various mutated elf4G alleles examined, only those resulting in in-frame mutations in SVLFPNLAGKS residues (mainly NL), adjacent to the YYV residues, conferred resistance. Furthermore, our data suggest that elf4G is essential for normal development, as alleles resulting in truncated elf4G could not be maintained in homozygous state. The final products with RTSV resistance and enhanced yield under glasshouse conditions were found to no longer contain the Cas9 sequence. Hence, the RTSV-resistant plants with the novel elf4G alleles represent a valuable material to develop more diverse RTSV-resistant varieties.

Introduction
Achieving high crop yields with efficient and sustainable agricultural practices is essential to attain global food security. Within the current context of climate change affecting the arable farm land and natural resources, the demand to produce sufficient agricultural products for the increasing human population is constantly expanding (Ronald, 2014). The escalation of biotic and abiotic stresses adds even more pressure to agricultural crop production. This especially applies in the case of rice as a species responsible for feeding billions of people, many of whom represent the low-income population from developing and underdeveloped countries in Asia and Africa (Seck et al., 2012).

The effort to increase rice production is often constrained by outbreaks of viral diseases. Among these, rice tungro disease (RTD) causes severe disruption of rice production, affecting more than 350 000 ha throughout the main rice-producing Asian countries (Azzam and Chancellor, 2002; Chancellor et al., 2006; Muralidharan et al., 2003). Rice plants affected by RTD show symptoms such as stunting or yellow discoloration of the leaves at the early stages, and reduced tillering and sterile panicles at later stages (Hull, 1996). RTD is caused by the interaction between Rice tungro spherical virus (RTSV), having a single-stranded RNA genome, and Rice tungro bacilliform virus (RTBV), having a double-stranded DNA genome (Hull, 1996). RTSV and RTBV are transmitted predominantly by green leafhopper (GLH) species such as Nephotettix virescens and N. nigropictus (Hibino and Cabauatan, 1987). RTBV is responsible for the development of the disease symptoms, while RTSV acts as a helper virus assisting transmission of RTBV by GLH and enhancing the symptoms (Hull, 1996). Cultivars with resistance to RTSV can contribute to a reduction in RTD in large fields, as plants infected with RTBV alone do not serve as sources for secondary spread (Anjaneyulu et al., 1994).

Extensive breeding programmes, conducted at International Rice Research Institute (IRRI), based on the screening of rice germplasm collections led to the identification of a number of rice cultivars resistant to RTD (Hibino et al., 1999; Khush et al., 2004; Sebastian et al., 1996). Several studies then focused on the Indica rice cultivar Utri Merah, which is resistant to both RTSV and RTBV (Azzam et al., 2001; Cabunagan et al., 1999; Ebron et al., 1994). Using near-isogenic lines (NILs) derived from Utri Merah, Encabo et al. (2009) demonstrated that the resistance to RTSV and RTBV in Utri Merah is an independent trait. RTSV resistance was found to be a recessive trait controlled by the translation initiation factor 4 gamma (elf4G) gene (Lee et al., 2010). By comparing the gene sequences in RTSV-resistant and RTSV-susceptible cultivars, the authors pinpointed that the single-nucleotide polymorphisms (SNPs) or deletion affecting the $Y^{1059V,1060V,1061V}$ amino acid residues were responsible for the resistant phenotype (Lee et al., 2010). Another report showed the involvement of elf(iso)4G in recessive resistance to Rice yellow mottle virus (RYMV) (Albar et al., 2006). The association of elf4G and elf(iso)4G with virus resistance is due to their essential roles in the assembly of basal translational initiation factors at the 5’-end of mRNA as well as in the cap-independent translation of viral RNA genomes (Kneller...
et al., 2006; Sonenberg and Hinnebusch, 2009). To undergo translation for synthesis of viral proteins, RNA viruses exploit the host cellular translational machinery to reprogramme translation to favour their own protein synthesis (Oreher and Miller, 2006).

In the past, traditional breeding techniques had been used to introduce various disease resistance genes from natural variants into valuable varieties. Nowadays, genome editing tools based on the activity of site-specific nucleases greatly facilitate the introduction of targeted mutations at designated genomic sites that are associated with disease resistance traits (Lee et al., 2016; Zhu et al., 2017). Among such genome editing tools, the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat-associated protein 9) system is gaining the highest interest, as it is the most user-friendly and highly efficient (Barakate and Stephens, 2016; Belhaj et al., 2013). With CRISPR/Cas9 targeted cleavage, small deletions or insertions are introduced at specific sites by harnessing the ability of nonhomologous end-joining (NHEJ) to repair the induced double-strand breaks (Belhaj et al., 2016). When engineering the plant host genome, genes for translation initiation factors eIF4E and eIF(iso)4E were mutated using the CRISPR/Cas9 system, resulting in the development of Arabidopsis lines resistant to Turnip mosaic virus (Pyott et al., 2016), and cucumber plants with broad-spectrum resistance to Cucumber vein yellowing virus, Zucchini yellow mosaic virus and Papaya ringspot mosaic virus (Chandrasekaran et al., 2016).

In this study, the CRISPR/Cas9 system was used to mutate the eIF4G gene in Oryza sativa var. indica cv. IR64, a widely grown cultivar across tropical Asia. IR64 is susceptible to RTSV (Hibino et al., 1988), possessing a susceptible (S)-type allele of eIF4G, whereas resistant cultivars able to suppress RTSV infection have the non-S-type allele of eIF4G with the nonsynonymous SNPs or deletion causing mutations at the Y1059V1060V1061 residues (Lee et al., 2010). Hence, we designed CRISPR/Cas9 reagents to target the sequences flanking this region of eIF4G with the aim to develop RTSV-resistant varieties that could potentially be released as nongenetically modified plants to the market whether/when proper regulatory policies are set in place. Our results showed that targeted mutagenesis mediated by CRISPR/Cas9 was highly efficient and dependent on the gRNA sequence, and the targeted mutations were inherited in subsequent generations. Importantly, some of the edited plants were resistant to RTSV, and the analysis of novel alleles of eIF4G (from the sequence SVLFPNLAGKS adjacent to the YVV residues) in the RTSV-resistant plants suggested that amino acid residues NL are also associated with RTSV resistance. The selected T2 plants were found to no longer contain the Cas9, and no off-target effects were evidenced. When the T2 plants were inoculated with RTSV, their yield was significantly higher than that of wild-type IR64 under glasshouse conditions.

Results

CRISPR/Cas9 efficiently generates variation in the rice eIF4G in T2 generation

To construct the CRISPR/Cas9 vectors, three gRNAs’ targeting sequences in the region between nucleotide positions 4140 and 4414 of eIF4G (Figure 1a, Table S1) were designed. The target locations were chosen based on the previous study showing that SNPs at positions 4387 and 4390 were associated with the reaction to RTSV (Lee et al., 2010). The gRNA1 was designed to target around 230 bp upstream of the indicated SNPs, gRNA2 was designed to target 10 bp upstream of this region, and gRNA3 was designed to target 5 bp downstream of the SNP4390.

An in silico analysis was performed on the DNA sequences of the three gRNAs to evidence the GC% and types of DNA bases in important positions (N-20 and N-3) (Liu et al., 2016) (Table S1). The GC contents (45%–55%) were in the accepted range (30%–80%) for plant-derived gRNAs (Liang et al., 2016). T at position N-3 and C at position N-20 were associated with increased cutting efficiency in animal cells (Liu et al., 2016), but not in plants (Liang et al., 2016). In our gRNAs, all three sequences possess T at position N-3, while C at position N-20 is present only in gRNA2, as the gRNA1 and gRNA3 have G at this position (Table S1).

The T-DNA in the final vectors pCas9-eIF4G-gRNA1, pCas9-eIF4G-gRNA2 and pCas9-eIF4G-gRNA3 contained three cassettes: (i) the Cas9 cassette to express a plant codon-optimized Cas9 from Streptococcus pyogenes driven by the Zea mays ubiquitin (ZmUBI1) promoter, (ii) the gRNA cassette to express one of the three gRNAs, each driven by the Triticum aestivum U6 promoter, and (iii) the plant selection cassette containing hyg (confering resistance to hygromycin) driven by the CaMV35S promoter (Figure 1b). The entire experimental scheme pursued after the vector construction is represented in Figure 2.

Agrobacterium-mediated transformation was performed using rice immature embryos, and the generated events were designated as 1146 when generated with pCas9-eIF4G-gRNA1, 1147

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**Figure 1** Gene editing of rice eIF4G. (a) Schematic illustration of the eIF4G locus (LOC_Os07g36940) composed of 12 exons (black boxes) and 11 introns (black lines); the YVV residues known to be associated with reactions to RTSV are indicated with a grey box and the gRNA target sequences are represented as red triangles. (b) Schematic diagrams of CRISPR/Cas9 vectors. TaU6, *Triticum aestivum* U6 promoter; ZmUB1, *Zea mays* ubiquitin promoter; Cas9, CRISPR-associated protein-9 nuclease gene; PolyA, termination signal; CaMV35S, *Cauliflower* mosaic virus 35S promoter; Hygromycin, hygromycin resistance gene.

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when generated with pCas9-eIF4G-gRNA2 and 1148 when generated with pCas9-eIF4G-gRNA3. The T0 events selected on hygromycin-containing media were first examined for the presence of T-DNA by PCR, and the overall transformation efficiency was 48.0% (Table 1). Subsequently, the T7 endonuclease I (T7EI) assay was performed to screen for the presence of mutations (Figure S1). Of the 72 transgenic events, 43 (59.7%) were positive for mutations in the T7EI assay (Table 1). The mutation rate for the individual constructs was 36.0% (pCas9-eIF4G-gRNA1), 86.8% (pCas9-eIF4G-gRNA2) and 65.6% (pCas9-eIF4G-gRNA3) (Table 1). All 43 events with putative mutations underwent sequencing analysis, and the observed types of mutations are presented in Table S2. The majority of the mutations in the T0 events were biallelic (27 of 43, 62.8%) or chimeras (15 of 43, 34.8%). Our results are similar to those from previous studies in rice (Li et al., 2013; Xu et al., 2015; Zhang et al., 2014). The event with the homozygous mutation (1148-16, with a five-nucleotide deletion) detected via sequencing analysis proved to be sterile (data not shown). Different types of mutations are present in the individual events, with most of them possessing more than just one type of mutation (Table S2). The most frequent were deletions (83.3%–100%), but substitutions (25.0%–45.0%) and insertions (5.0%–58.3%) were also present (Table S3). When considering each type of mutation, 16 (37.2%) T0 events contained just deletions, whereas 15 (34.8%) events contained a combination of deletions and substitutions. Moreover, three (6.9%) events (1148-06, 1147-03 and 1147-09) had a combination of deletions, substitutions and insertions, with no event with just insertions or just substitutions was obtained (Table S2). The number of bases affected ranged from 1 to 3 for insertions and substitutions, and from 1 to 63 for deletions, with more than 50% of the events showing deletions longer than 5 bp (Table S2).

These observations are in line with the previous studies performed in rice (Li et al., 2017; Miao et al., 2013; Xu et al., 2015; Zhang et al., 2014), although we obtained higher rates of insertions and substitutions. Alignments of the targeted sequence in IR64 and mutated sequences in selected T0 events are presented in Figure 3a and selective chromatograms are provided in Figure S2.

The predicted protein sequences from the mutated eIF4G alleles were determined in selected T0 events to evaluate the effect of mutations at this level. All of the selected events have one allele (A1) predicted to encode eIF4G of nearly full length with one to seven amino acids deleted, while the second allele (A2) is predicted to encode a truncated eIF4G (Figures 3b, 4). The predicted truncated eIF4G variants can be divided into three categories: (i) truncated upstream of the YVV residues (events 1146-12, 1146-22); (ii) truncated with YVV residues mutated to LCC (events 1147-03, 1147-10, 1147-11, 1147-13) and (iii) truncated downstream of the YVV residues (events 1148-05, 1148-20) (Figure 4). Events 1146 were generated with pCas9-eIF4G-gRNA1, which targets the nucleotides translated to the VNMGED sequence (amino acids 1008-1013) located 76 amino acids upstream of the YVV sequence (amino acids 1060-1062). The predicted protein sequence in events 1146-12 and 1146-22 shows that the VNMGED sequence was mutated in both alleles (Figure 3b). The predicted products from one of the alleles in both events were truncated upstream the YVV sequence (Figure 4). In the events generated with pCas9-eIF4G-gRNA2 construct (1147), gRNA2 targets the nucleotides translated to the VLPNL sequence (amino acids 1050-1155) located five amino acids upstream of the YVV sequence. The predicted protein sequences showed mutated amino acid residues within or between the SVLPNLAGKSYVV sequence (mainly the NL residues) in one allele, while the predicted proteins in the second allele were truncated and had the YVV sequence mutated to LCC.

### Table 1

Transformation efficiency and mutation rate in T0 events generated by the Agrobacterium-mediated transformation of immature embryos with CRISPR/Cas9 constructs

| Vector ID | Vector description | No. of immature embryos | No. of regenerated plants | No. of transgenic events | Transformation efficiency (%) | No. of events with mutations | Mutation rate (%) |
|-----------|--------------------|--------------------------|---------------------------|--------------------------|-------------------------------|-----------------------------|-------------------|
| 1146      | pCas9-eIF4G-gRNA1  | 50                       | 25                        | 25                       | 50.0                          | 9                           | 36.0              |
| 1147      | pCas9-eIF4G-gRNA2  | 50                       | 15                        | 15                       | 30.0                          | 13                          | 86.6              |
| 1148      | pCas9-eIF4G-gRNA3  | 50                       | 32                        | 32                       | 64.0                          | 21                          | 65.6              |
| Total     |                    | 150                      | 72                        | 72                       | 48.0                          | 43                          | 59.7              |
Lastly, in the events generated with pCas9-eIF4G-gRNA3 construct (1148) , gRNA3 targets nucleotides translated to the DHPSPG sequence (amino acids 1063-1069) located immediately downstream of the YVV sequence. Disruption of the DHPSPG sequence was observed in the products predicted from both alleles in two T₀ events, 1148-5 and 1148-20, but the YVV sequence in the predicted product remained intact (Figure 3b). The infertile T₀ event 1148-16 contains homozygous eIF4G alleles with five nucleotides deleted. This deletion was predicted to result in mutations of DHPSPG to DHPRKG without affecting the YVV sequence, but creating a premature stop codon immediately after DHPRKG (Figure S3). This suggests that eIF4G is essential for plant growth as plants in which both eIF4G alleles produced truncated proteins had sterile seeds, while the presence of an allele encoding a nearly full-length eIF4G with localized mutations seemed to be sufficient to sustain normal plant growth and seed development.

Novel eIF4G alleles are transmitted to T₁ and T₂ generations with no detectable mutations in the closest off-target site

Several T₁ progenies generated from selected T₀ events underwent a T7E1 assay and genotyping analyses to investigate the pattern of transmission of CRISPR/Cas9-mediated targeted gene modifications. As expected for biallelic and chimeric T₀ events containing T-DNA, we observed that some mutations were lost in the T₁ generation (e.g. 1146-12 and 1147-03), whereas some new mutations occurred as well (e.g. 1147-05) (Table S4). Based on genotyping analyses, among the 24 T₁ plants analysed, just one (1148-05) was homozygous, while the rest had biallelic mutations. The chimeric mutations found in T₀ events were no longer present in T₁ plants. The presence/absence of Cas9 was determined by PCR, and 17 of 24 events (70.8%) were negative, while 7 (29.2%) were positive for Cas9 (Table S4). The segregation ratios of mutations in T₁ plants showed that generally these did not follow a Mendelian distribution (Table S5). Similar observations with regard to loss/acquisition of mutations and distorted inheritance pattern in the T₁ population were also reported in previous studies (Pan et al., 2016; Xu et al., 2015).
Seeds from selected T1 plants with biallelic mutations and with or without the Cas9 transgene were sown to generate the T2 population. All T2 plants examined were negative for Cas9 (Table S4). The expected segregation pattern for biallelic mutations was 1 (homozygous for mutation 1) : 2 (heterozygous) : 1 (homozygous for mutation 2) (Feng et al., 2014; Xu et al., 2015). However, one of the alleles was predominantly lost and most segregation ratios were 1:2:0 or 1:1:0 (Table S5). The distorted segregation of mutations in both T1 and T2 populations suggests that the two alleles are not inherited with equal frequencies (Pan et al., 2016; Zhang et al., 2014). One of the reasons for the distorted segregation could be that the alleles that were lost more often were those encoding a truncated protein, as the presence of functional eIF4G seems to be essential for plant development as addressed above.

As low-frequency cases of off-target cleavage by the CRISPR/Cas9 system have been reported in plants (Shan et al., 2013; Zhang et al., 2014), we examined the presence of potential off-target mutations in selected T2 plants of the 1147 lines (pCas9-eIF4G-gRNA2 construct). We performed the analysis in the T2 plants as these no longer contain Cas9, and hence, no additional cuts would be generated. Potential off-target loci were predicted based on the protospacer adjacent motif (PAM) sequence using the CRISPR-P online tool (Lei et al., 2014). Three potential sites containing three to four mismatched bases were retrieved (Figure S4). We chose to further analyse the site with three mismatches (Chr11:17936212) representing the closest off-target. DNA was extracted from 23 randomly selected T2 plants of the 1147 lines and subjected to PCR and sequencing analyses. No mutations were found in the putative off-target site in any of the T2 plants examined (Figure S4), underlining the high specificity of CRISPR/Cas9-induced mutagenesis.

In-frame mutations in eIF4G confer RTSV resistance in T2 and T3 population

T2 plants generated from selected T1 events were evaluated for RTSV resistance to assess the association between the eIF4G mutations and the reaction to RTSV. As plants at the early seedling stage are most susceptible to RTSV (Rao and Anjaneyulu, 1980), we inoculated 10-day-old T2 plants with RTSV via GLH for 24 h. The mutation type, zygosity, protein sequence alignment and reactions to RTSV of selected T2 plants are shown in Data S1. The results of enzyme-linked immunosorbent assay (ELISA) using an RTSV-specific antibody showed that all T2 plants generated from 1147 events obtained using the pCas9-eIF4G-gRNA2 construct were resistant to RTSV (Tables S4, S6). The T2 plants from 1147 events resistant to RTSV have one allele encoding eIF4G of nearly full length with in-frame mutations in SLYFPNLAGKS residues located immediately upstream of the YVV residues, while the other allele encodes a truncated eIF4G with the YVV residues mutated to LCC (Figures 3b, 4). On the other hand, the reactions to RTSV of T2 plants derived from 1146 and 1148 events were inconsistent, showing resistance or susceptibility (Tables S4, S6). All of the T2 plants from 1146 and 1148 events have one allele encoding eIF4G of nearly full length with mutations located about 76 amino acids upstream of the YVV residues or immediately downstream of the YVV residues and possessing intact YVV residues (Figures 3b, 4).

To better understand the relationship between mutated eIF4G alleles and the observed RTSV phenotype, we grouped the alleles with mutations in the following four categories: (i) A-type alleles encode eIF4G with amino acid substitutions/deletions about 76 amino acids upstream of the YVV residues, as in the case of 1146 events derived from the pCas9-eIF4G-gRNA1 construct; (ii) B-type alleles encode eIF4G with changes in NLS residues, upstream of the YVV residues, as in the case of 1147 events derived from the pCas9-eIF4G-gRNA2 construct; (iii) C-type alleles encode eIF4G with substitutions/deletions immediately downstream of the YVV residues, as in the case of 1148 event derived from the pCas9-eIF4G-gRNA3 construct; and (iv) D-type alleles encode a truncated eIF4G. All T2 plants from 1147 events with a B/B or a B/D allele combination were resistant to RTSV (Figure 5, Data S1). On the other hand, the reactions to RTSV of T2 plants from 1146 and 1148 events carrying an A/A, A/D, C/C or C/D allele combination were inconclusive, showing resistance or susceptibility (Figure 5, Table S6, Data S1). As mentioned above, plants with a D/D allele combination are not likely to be viable, and we were not able to observe any T2 plants with a D/D allele combination.

To further confirm the stability of the trait, selected T2 events were advanced to the T3 generation and selected T3 plants were evaluated for reactions to RTSV. All T3 plants from 1147 events were resistant, whereas the reactions of T3 plants derived from 1146 and 1148 events were still inconclusive, showing susceptibility or resistance to RTSV (Table S7). Sequencing analysis for the eIF4G alleles in the T3 plants from 1147 events showed that all of the plants have either B/B or B/D alleles (Table S8). Collectively, these results indicate that, among the mutated eIF4G alleles generated by the CRISPR/Cas9 system, only the eIF4G

**Table 5** Reactions of the edited plants to RTSV depending on the type and zygosity of mutations. The novel eIF4G alleles are classified into four groups: A-type allele resulting in substitutions/deletions about 76 amino acids upstream of the YVV residues; B-type allele resulting in substitutions/deletions immediately upstream of the YVV residues; C-type allele resulting in substitutions/deletions downstream of the YVV residues; and D-type allele resulting in premature stop codon. The observed phenotypes are A/A and A/D — inconclusive (resistant or susceptible), B/B and B/D — resistant, C/C and C/D — inconclusive (resistant or susceptible), and D/D — plant not viable.
alleles resulting in in-frame mutations in the SVLFPNLAGKS sequence (mainly the NL residues) immediately upstream of the YVV residues can explicitly confer resistance to RTSV.

Agronomic parameters such as plant height, numbers of panicles and filled grains, and total filled-grain weight were measured in the RTSV-inoculated T2 plants from 1147 events and RTSV-inoculated and mock-inoculated IR64 plants (Table 2, Figure S5). When T2 plants from 1147 events were inoculated with RTSV, their agronomic parameters were significantly higher than those of the RTSV-inoculated IR64 control plants under glasshouse conditions.

Discussion

The development of rice varieties with resistance to RTD has been a major objective for breeding programmes in tropical Asian countries. Genotyping and phenotyping for RTD resistance of a great number of rice accessions have been carried out in the last 50 years (Azzam et al., 2000). The information gathered from previous studies on RTD resistance and the technical advances provided by the development of genome editing tools had set the stage for the current study.

Plant viruses need host factors to propagate their genomes in host cells. This dependence can be harnessed to develop crops with virus resistance. To this end, an effective approach is to produce targeted mutations in genes that encode a host factor critical for the viral infection process. Genome editing is a useful tool that can shorten the time needed to replace alleles and develop plants with specific traits (Puchta, 2017; Weeks et al., 2015). In our case, the development of RTSV-resistant rice was achieved in a considerably shorter period than with traditional breeding. The elf4G gene targeted in this study is responsible for resistance to RTSV (Lee et al., 2010). Using three different gRNAs targeting regions surrounding the nucleotide sequence encoding the YVV residues associated with RTSV resistance (Lee et al., 2010), we obtained novel elf4G alleles with high efficiency. The types of mutations obtained in T0 events, zygosity and transmissibility of the mutations to successive generations are in agreement with other studies performed in rice (Li et al., 2017; Miao et al., 2013; Xu et al., 2015; Zhang et al., 2014). The distorted segregation pattern of mutations (1:2:0 or 1:1:0) in the T2 generation could be explained, at least in part, by the presence of alleles that result in truncation of elf4G. The elf4G function seems to be essential for plant development, and homozygotes for alleles encoding a truncated elf4G are most likely to be lethal as we did not observe T2 or T3 plants with homozygous alleles for a truncated elf4G. This finding provides a new insight into the function of rice elf4G. Albeit the presence of additional genes for elf4G isoforms in the rice genome, which are thought to have a partial functional redundancy enabling to compensate the loss of elf4G (Sanfaçon, 2015), our study shows that this is not likely to be the case. Finally, the T2 mutant plants no longer contained Cas9 transgene and no off-target effects were observed, which are important criteria to avoid the regulatory constraints in commercialization/release of transgenic plants (Huang et al., 2016; Voytas and Gao, 2014).

Lee et al. (2010) and Leung et al. (2015) mentioned that the YVV residues are critical to determining whether elf4G is associated with susceptibility or nonsusceptibility (resistance) to RTSV. The most interesting observation revealed in our study is that, as seen in RTSV-resistant T2 and T3 plants from 1147 events, mutations within the SVLFPNLAGKS sequence (mainly the NL residues) located immediately upstream of the YVV residues appear to be associated with the reaction to RTSV. All RTSV-resistant T2 and T3 plants from 1147 events can be categorized in two classes: (i) plants with the homozygous elf4G allele containing in-frame mutations that result in changes in at least two of the amino acid residues in SVLFPNLAGKS (e.g. NL residues) and (ii) biallelic plants with one allele containing in-frame mutations resulting in changes in NL residues and the second allele containing mutations resulting in premature termination of elf4G (Data S1). This finding underlines the presence of additional amino acid residues associated with RTSV resistance that can be modified to create novel elf4G alleles conferring resistance to RTSV and thus broadening the range of possibilities for developing RTSV-resistant varieties. On the other hand, the reaction to RTSV of the T2 plants from 1146 and 1148 events was inconclusive (Tables S4, S6, Data S1). The in-frame mutations found in the T2 plants from the 1146 events and those from the 1148 events occurred further upstream or immediately downstream of the YVV (and SVLFPNLAGKS) residues. The inconclusive reactions could be a consequence of (i) minor effects on the reactions to RTSV by the mutations, (ii) moderate GLH resistance of IR64 that might prevent successful transmission of RTSV (Shibata et al., 2007; Zenna et al., 2008) or (iii) combination of both. In any case, the amino acids mutated in a nearly full-length

![Table 2 Agronomic parameters measured in selected T2 plants grown under glasshouse conditions](image)

| T2 events | No. of T2 plants | Plant height (cm) | No. of panicles | No. of filled grains | Total filled grains weight (g) |
|-----------|-----------------|------------------|----------------|--------------------|-------------------------------|
| 1147-03-10 | 20 | 118.7±5.5 | 3.2*** | 2.7*** | 254.1*** |
| 1147-10-12 | 13 | 117.9±4.3 | 2.8*** | 2.3*** | 248.2*** |
| 1147-10-19 | 12 | 116.0±5.3 | 3.2*** | 2.8*** | 240.1*** |
| 1147-10-20 | 15 | 118.7±4.5 | 3.2*** | 2.7*** | 251.3*** |
| IR64 WT RTSV | 15 | 117.9±4.3 | 2.8*** | 2.3*** | 249.1*** |
| IR64 WT CTRL | 8 | 119.5±4.9 | 3.2*** | 2.7*** | 254.1*** |

*Data are expressed as means ± standard deviation. Results were subjected to analysis of variance, and the means were compared by Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001) using IR64 WT RTSV as the reference control. †IR64 WT RTSV represents the nontransgenic control subjected to RTSV inoculation. ‡IR64 WT CTRL represents the nontransgenic untreated control (mock-treated).*

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elf4G of the T_2 plants from 1146 and 1148 events are most likely not to have a significant effect on the reactions to RTSV. The amino acid residues most commonly mutated in elf4G of the RTSV-resistant T_2 plants from 1147 events were the NL residues within the SVLFPNLAGKS sequence. Thus, in addition to the YVV residues, the NL residues are likely to be other key residues determining the reaction of rice to RTSV. The biological function of SVLFPNLAGKSYYVV residues in determining the resistance of rice plants to RTSV is still to be investigated. It was reported that mutations in the MIF4G domain (Ponting, 2000) of elf(iso)4G, without affecting the structure of MIF4G, resulted in the loss of interaction between elf(iso)4G and the VPg (viral protein genome-liked) protein of RYMV and, subsequently, the loss of susceptibility of rice to RYMV (Hébrard et al., 2010). The SVLFPNLAGKSYYVV residues in the rice elf4G are located between the elf4E-binding motif (Mader et al., 1995) and the MIF4G domain, both of which are essential to the function of elf4G. Therefore, it is reasonable to conjecture that the SVLFPNLAGKSYYVV residues are potentially involved in the interaction between elf4G and an RTSV protein that affects the reaction of rice to RTSV. In summary, here we report on the development of IR64-derived rice lines with resistance to RTSV by CRISPR/Cas9-mediated targeted mutagenesis of elf4G. The induced mutations were successfully transmitted to the next generations. As a recessive trait, homozygous lines are required for resistance, but in our study, biallelic mutants where only one functional mutated allele was present and the other allele encodes a truncated nonfunctional protein also showed the desired phenotype. Moreover, our results demonstrated that, in addition to the YVV residues (Lee et al., 2010; Leung et al., 2015), the NL residues within the SVLFPNLAGKS sequence of elf4G are also critical to determine the reaction to RTSV and that in-frame mutations in this sequence resulted in resistance to RTSV. The final products show resistance to RTSV without detectable off-target mutations or Cas9 insertion in the genome. Therefore, the RTSV-resistant plants generated in this study may represent a valuable material as alternative sources of RTSV resistance and plants of subsequent generations (T_1, T_2 and T_3) using the CTAB method as previously described (Stewart and Via, 1993). To identify the presence of the T-DNA in the rice genome, PCR was conducted using primers for Hyg (F1 and Hyg R1), while the presence/absence of Cas9 was assessed using the primer pair Tacas9_1F and Tacas9_1R (Table S9). The PCR conditions were as follows: denaturation at 95 °C/5 min; 35 cycles of 95 °C/30 s, 55 °C/30 s and 72 °C/2 min; and a final extension at 72 °C/5 min. Positive transgenic events were screened for mutations using the T7EI assay. As a first step, PCR products (of about 1.5 kb) encompassing the gRNA target sites were generated using the genomic DNA as template with primers elf4Gtf and elf4Gtr (Table S9) and Q5 DNA polymerase (New England Biolabs). In the second step, 200 ng of purified PCR products were denatured and re-annealed in a PCR machine, and consecutively digested with 1U of T7 endonuclease I (New England Biolabs); the conditions used were: denaturation at 95 °C/5 min; 35 cycles of 95 °C/30 s, 55 °C/30 s and 72 °C/2 min; and a final extension at 72 °C/5 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). In the second step, 200 ng of purified PCR products were denatured and re-annealed in a PCR machine, and consecutively digested with 1U of T7 endonuclease I (New England Biolabs); the conditions used were as follows: 95 °C/5 min, 85 °C/5 min, 25 °C/5 min and 37 °C/1 h. As a last step, 0.25 μM EDTA was added to stop the reaction and the fragments were subjected to electrophoresis on an 1% agarose gel. Three independent PCRs for each positive sample were sequenced directly without cloning (Macrogen). The sequences were analysed using DSDecodeM (http://skl.scau.edu.cn/dsdecode/) to decode the superimposed sequencing chromatograms and CRISPR-ID (http://crispid.biomed.kuleuven.be/) to detect the size and localization of indels.

Experimental procedure

Design of CRISPR/Cas9 vectors

The IR64 elf4G sequence was obtained from the SNP-seek database (Mansueto et al., 2017). The gRNA targets in the elf4G gene were selected using the E-CRISP Design Tool (Heigwer et al., 2014). The Cas9 expression vectors were constructed as described in Cermák et al. (2017) using protocols 2A and S. A version of pMOD_A1110 (ZmUbi:TaCas9—codon-optimized for wheat) with the octopine synthase terminator and an additional NLS and a FLAG tag at the C terminus of Cas9, along with pMOD_B2518 (TaU6:gRNA), pMOD_C0000 (empty), and the pTRANS_240 transformation backbone (T-DNA, 2x35S:hptII w/intron for selection) were used. The gRNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa) (Table S1). The resulting vectors (pcas9-elf4G-gRNA1, pcas9-elf4G-gRNA2 and pcas9-elf4G-gRNA3) were sequence-verified (Macrogen, Seoul, Korea) to confirm the presence of all elements.

Agrobacterium-mediated transformation

The pcas9-elf4G-gRNA1, pcas9-elf4G-gRNA2 and pcas9-elf4G-gRNA3 constructs were introduced into the virulent LBA4404 strain of Agrobacterium tumefaciens using the freeze/thaw method (Höfgen and Willmitzer, 1988). Immature embryos of Oryza sativa var. indica cv. IR64 were used for the Agrobacterium-mediated transformation, as previously described (Slamet-Loedin et al., 2014). For each construct, 50 immature embryos were used per transformation. The resulting regenerated plantlets were acclimatized to glasshouse conditions. The T_0 seeds were harvested and used to generate the T_1 population. Subsequently, T_1 seeds were used to generate the T_2 population and T_2 seeds to generate the T_3 population.

Detection of mutations

Genomic DNA was extracted from wild-type (WT) IR64, T_0 events and plants of subsequent generations (T_1, T_2 and T_3) using the CTAB method as previously described (Stewart and Via, 1993). To identify the presence of the T-DNA in the rice genome, PCR was conducted using primers for hyg (F1 and Hyg R1), while the presence/absence of Cas9 was assessed using the primer pair Tacas9_1F and Tacas9_1R (Table S9). The PCR conditions were as follows: denaturation at 95 °C/5 min; 35 cycles of 95 °C/30 s, 55 °C/30 s and 72 °C/2 min; and a final extension at 72 °C/5 min. Positive transgenic events were screened for mutations using the T7EI assay. As a first step, PCR products (of about 1.5 kb) encompassing the gRNA target sites were generated using the genomic DNA as template with primers elf4Gtf and elf4Gtr (Table S9) and Q5 DNA polymerase (New England Biolabs (Ipswich, Massachusetts)). The PCR conditions were as follows: denaturation at 95 °C/5 min; 35 cycles of 95 °C/30 s, 55 °C/30 s and 72 °C/2 min; and a final extension at 72 °C/5 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). In the second step, 200 ng of purified PCR products were denatured and re-annealed in a PCR machine, and consecutively digested with 1U of T7 endonuclease I (New England Biolabs); the conditions used were as follows: 95 °C/5 min, 85 °C/5 min, 25 °C/5 min and 37 °C/1 h. As a last step, 0.25 μM EDTA was added to stop the reaction and the fragments were subjected to electrophoresis on an 1% agarose gel. Three independent PCRs for each positive sample were sequenced directly without cloning (Macrogen). The sequences were analysed using DSDecodeM (http://skl.scau.edu.cn/dsdecode/) to decode the superimposed sequencing chromatograms and CRISPR-ID (http://crispid.biomed.kuleuven.be/) to detect the size and localization of indels.
evaluated for virus infection by ELISA at 21 days postinoculation (dpi) as described by Cabauatan et al. (1995) using the extracts from the second youngest leaf collected from each plant. The presence of RTSV in the leaf extract was determined by measurement of the absorbance at 405 nm using an ELISA reader (BioTek Instruments, Winooski, Vermont). Samples with an absorbance value >0.1 were considered positive for RTSV infection.

Agronomic parameters

T₂ plants from selected T₁ seeds were inoculated with RTSV and grown in the glasshouse until maturity to determine several agronomic parameters. Agronomic phenotypes such as plant height, panicle count, filled-grain number and total filled-grain weight were measured upon harvest. Plant height was measured from the base of the stalk to the tip of the highest panicle of the highest tiller. Seed count and seed weight were acquired upon processing in the transgenic seedatory laboratory. Data are expressed as means ± standard deviation values. Results were subjected to statistical analysis by Student’s t-test (\( *P < 0.05, **P < 0.01, ***P < 0.001 \)).

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Authors’ contributions

PCM and IRC conceptualized the study and designed the experiments; TC and DFV designed the vectors; CC generated the T₀ population; ISL supervised the plant experiments; TC and DFV designed the vectors; CC generated and analysed the T₁, T₂ and T₃ populations; and AM, PCM and IRC performed the RTSV phenotyping; NRS generated and characterized the T₁, T₂ and T₃ populations; and AM, PCM and IRC supervised and managed the laboratory. ACR and AGM, PCM and IRC performed the statistical analysis by Student’s t-test.

Conflict of interest

The authors declare no competing financial interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Screening for mutations in T0 population. (a) Gel images of PCR reactions using primers (elf4FGit and elf4FGitR) flanking the targeted region. (b) Gel images of T7E assay performed on amplified bands. Numbers in red represent samples positive for mutations as evidenced by the presence of double bands in T7E assay. L, 1 kb ladder; Ctrl, controls; C+, positive control (wild-type DNA samples); C−, negative control (no DNA control); C+ d, wild-type digested; C+ n, wild-type nondigested Fig 25 Representative chromatograms of selective events from T0 population. The sequencing results provided by Macrogen were analyzed using DSDecode (http://skl.scau.edu.cn/dsdecode) to decode the superimposed sequencing chromatograms, and CRISPR-ID (http://crispid.biomed.kuleuven.be/) to detect the size and localization of indels

Figure S3 Example of a T0 event (1148–16) with a homozygous allelence encoding a truncated elf4F. Even if the YVV sequence is not affected, the truncated protein from both alleles resulted in a sterile plant

Figure S4 Examination for the presence of mutations in the putative off-target sites. The analysis was conducted in T2 generation derived from 1147 (gRNA2A) lines. Three different putative off-target sites were picked up by CRISPR-P (http://cbi.hzau.edu.cn/crispr/) in an in silico analysis. The most probable off-target site (OFF1) was selected to confirm the in silico results by PCR and Sanger sequencing. The alignment between WT and selected T2 mutated events (homozygous and biallelic) is shown and the putative target is evidenced in red

Figure S5 Selected 90-day-old T2 plants at 80-days post-inoculation with RTSV. Ten-day-old mutated plants and respective controls (IR64 WT, non-transformed TW16) were inoculated with RTSV via GLH and subsequently grown under greenhouse conditions

Table S1 Analysis of gRNA sequences used for CRISPR/Cas9 vector construction

Table S2 Type of mutations in T0 events obtained by transformation with three CRISPR/Cas9 constructs

Table S3 Percentage of deletions, insertions, and substitutions found in T0 events

Table S4 Type of mutations transmitted to T1 and T2 plants, and reactions to RTSV of T0 plants derived from T0 events generated by the three CRISPR/Cas9 constructs

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Table S5 Pattern of mutation transmissibility in T₁ and T₂ populations

Table S6 RTSV phenotype of selected lines from T₂ population. TW16 and Taichung Native 1 (TN1) were used as the resistant and susceptible controls, respectively.

Table S7 RTSV phenotype of selected lines from T₃ population. TW16 and Taichung Native 1 (TN1) were used as the resistant and susceptible controls, respectively.

Table S8 Genotype of eIF4G and reaction to RTSV in selected T₃ plants.

Table S9 Primer sequences used in the present study.

Data S1 Genotype and reaction to RTSV of T₂ lines. Nucleotide and protein alignments are presented compared to IR64 wild type eIF4G sequence. R, resistant; S, susceptible; A, A-type allele resulting in substitutions/deletions about 76 amino acids upstream of the YVV residues; B, B-type allele resulting in substitutions/deletions immediately upstream of the YVV residues; C, C-type allele resulting in substitutions/deletions downstream of the YVV residues; D, D-type allele resulting in premature stop codon. The observed phenotypes are: A/A and A/D - inconclusive (resistant or susceptible), B/B and B/D - resistant, C/C and C/D - inconclusive (resistant or susceptible).