Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2019) **17**, pp. 2096–2105 doi: 10.1111/pbi.13125

CRISPR/Cas9-mediated functional recovery of the recessive *rc* allele to develop red rice

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Received 15 December 2018;
revised 24 March 2019;
accepted 29 March 2019.
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Keywords: CRISPR/Cas9, functional recovery, *rc*, red rice, proanthocyanidins.

Summary

Red rice contains high levels of proanthocyanidins and anthocyanins, which have been recognized as health-promoting nutrients. The red coloration of rice grains is controlled by two complementary genes, *Rc* and *Rd*. The *RcRd* genotype produces red pericarp in wild species *Oryza rufipogon*, whereas most cultivated rice varieties produce white grains resulted from a 14-bp frame-shift deletion in the seventh exon of the *Rc* gene. In the present study, we developed a CRISPR/Cas9-mediated method to functionally restore the recessive *rc* allele through reverting the 14-bp frame-shift deletion to in-frame mutations in which the deletions were in multiples of three bases, and successfully converted three elite white pericarp rice varieties into red ones. Rice seeds from T_1 in-frame *Rc* lines were measured for proanthocyanidins and anthocyanidins, and high accumulation levels of proanthocyanidins and anthocyanidins were observed in red grains from the mutants. Moreover, there was no significant difference between wild-type and in-frame *Rc* mutants in major agronomic traits, indicating that restoration of *Rc* function had no negative effect on important agronomic traits in rice. Given that most white pericarp rice varieties are resulted from the 14-bp deletion in *Rc*, it is conceivable that our method could be applied to most white pericarp rice varieties and would greatly accelerate the breeding of new red rice varieties with elite agronomic traits. In addition, our study demonstrates an effective approach to restore recessive frame-shift alleles for crop improvement.

Introduction

Rice (*Oryza sativa* L.) is a major staple food crop, feeding more than half the world's population. The majority of rice varieties grown and consumed throughout the world have white pericarp. There are also varieties of rice with brown, red or purple/black pericarp. The pigments of coloured rice contain high levels of proanthocyanidins and anthocyanins which have been recognized as health-promoting nutrients (Finocchiaro *et al.*, 2007; Gunaratne *et al.*, 2013; Qiu *et al.*, 2010). In China, Japan, Korea and many other countries in South-East Asia, coloured rice has been widely cultivated and consumed owing to its nutritional properties and health benefits.

Red pericarp is ubiquitous in wild rice species (*Oryza rufipogon* L.), which are the ancestors of cultivated rice. The red coloration in the grains of wild rice is controlled by two complementary genes, *Rc* and *Rd* (Furukawa *et al.*, 2007; Sweeney *et al.*, 2006). *Rc* encodes a basic helix–loop–helix (bHLH) transcription factor, whereas *Rd* encodes a dihydroflavonol-4-reductase (DFR) protein (Furukawa *et al.*, 2007; Sweeney *et al.*, 2006). The *RcRd* genotype produces red pericarp in *O. rufipogon*. In contrast, most cultivated rice varieties produce white grains, and about 97.9% of these are resulted from a 14-bp frame-shift deletion in the seventh exon of the *Rc* gene (Sweeney *et al.*, 2007). These previous studies demonstrated the critical role of *Rc* in determining the red coloration in rice grains. Thus, the *Rc* locus could be a genome editing target for developing red rice.

Genome editing technologies, especially the CRISPR/Cas9 system, have emerged as powerful tools to precisely breed crops with improved agronomic traits, such as growth period (Cai *et al.*, 2018), disease resistance (Macovei *et al.*, 2018; Wang *et al.*, 2014, 2016, 2018b), genic male sterility (Zhou *et al.*, 2016), nutrition quality (Clasen *et al.*, 2015) and grain yield (Li *et al.*, 2016; Wang *et al.*, 2018a). Currently, the common application of the CRISPR/Cas9 technology in crop breeding is to edit genes that have deleterious effects on agronomic traits. For instance, CRISPR/Cas9-mediated knockout of the *Waxy* gene was reported to reduce the amylose content in rice grains and could, thereby, convert common rice into glutinous rice (Zhang *et al.*, 2018). Given the fact that the CRISPR/Cas9 system could produce different types of mutations—deletion, insertion or substitution with lengths from 1 to 3 bases or longer (Zhang *et al.*, 2014, 2016; Zhou *et al.*, 2014)—we hypothesized that this technology could be applied to recover recessive alleles of positive regulatory genes lost during domestication, such as the *rc* allele, by reverting the frame-shift mutations to in-frame versions in which the lengths of deletions or insertions are in multiples of three bases.

In this study, we selected three elite rice varieties with white pericarp, including a *japonica* variety Xiushui134 (X134), an *indica* restorer line Shuhui143 (S143) and an *indica* photo-/thermo-sensitive genic male sterile line ZhiNongS (ZNS), to test our hypothesis. A total of 23 T_0 plants that contained at least one in-frame allele of the *Rc* gene were identified from CRISPR/Cas9

transgenic plants in all three varieties, and rice plants harbouring in-frame *Rc* alleles exhibited red coloration. Seeds from T_1 in-frame *Rc* lines were measured for proanthocyanidins and anthocyanidins, and high accumulation levels of proanthocyanidins and anthocyanidins were observed in red rice grains. Moreover, there was no significant difference between wild-type and in-frame *Rc* mutants in major agronomic traits, indicating that restoration of *Rc* function had no negative effect on important agronomic traits in rice. Thus, our method could be applied to most white pericarp rice varieties and would greatly accelerate the breeding of elite red rice varieties with high proanthocyanidins and anthocyanins.

Results

Site-specific mutagenesis of the *rc* allele mediated by CRISPR/Cas9 system

We first sequenced the *Rc* and *Rd* loci in X134, S143 and ZNS, and confirmed that all three varieties contained the same 14-bp deletion in the seventh exon of the *rc* allele (Figure S1). There were no mutations in the coding regions of the *Rd* gene (Figure S2). To achieve functional recovery of the recessive *rc* allele, two Cas9/sgRNA constructs targeting the flanking sequences of the 14-bp deletion site were designed (Figure 1a). The two constructs were transformed into *Agrobacterium tumefaciens* EHA105. Rice calli were co-cultured with *Agrobacterium* strains harbouring the two Cas9/sgRNA constructs. A total of 12, 26 and 22 independent T_0 transgenic plants were generated from the calli of X134, S143 and ZNS, respectively (Table 1). The genotyping of T_0 transgenic plants identified 9, 20 and 17 plants with mutations in the target sites in X134, S143 and ZNS, respectively (Tables 1 and S1). Surprisingly, among the T_0 mutant plants, 45.6% (21/46) were putatively homozygous and 52.2% (24/46) were bi-allelic, whereas only one plant (2.2%) was heterozygous (Tables 1 and S1). The reason for low percentage of the heterozygous genotype could be that the plants were generated by co-transformation of two sgRNA cassettes.

T_0 transgenic rice plants harbouring in-frame *Rc* variants exhibited red coloration

We aimed to screen plants with mutations that could revert the 14-bp deletion to a deletion in multiples of 3 bases. A total of 23 T_0 transgenic plants that contained at least one in-frame allele of the *Rc* gene were identified in all three varieties (Figure 1b, Tables 1 and S1). Most in-frame mutants carried a newly introduced 1-bp deletion adjacent to the 14-bp deletion site, resulting in deletions of a total of 15 bases (Figure 1b, Table S1). Sequence analysis showed that these in-frame variants encoded putative full-length polypeptides. Compared to the wild-type Rc protein in *O. rufipogon, these* polypeptides had minor deletions ranging from 5 to 10 amino acids. The polypeptides, however, contained an intact bHLH domain (Figures 1c and S3). Thus, minor deletions of noncritical residues outside the conserved bHLH domain should have no deleterious effects on Rc function.

In T_0 X134 and S143 plants harbouring in-frame *Rc* variants, the coloration of the grains changed from white to red (Figure 2), indicating that *Rc* gene function was successfully restored. We also observed that the red pericarp phenotype of the bi-allelic plants with one dose of in-frame *Rc* was similar to that of plants with a homozygous in-frame *Rc* genotype. Hence, similar to their wild-type *Rc* gene, these in-frame variants had a dominant effect on the red coloration in rice grains. As for T_0 ZNS plants, the mutants displayed an identical male sterility phenotype as the wild-type ZNS (Figure S4). We failed to harvest seeds from T_0 ZNS mutant plants in the summer season in Fuzhou. Instead, the ZNS mutant plants were crossed with a white pericarp restorer line MH86. As expected, the resulting hybrid seeds from the in-frame *Rc* ZNS mutants showed red grain pericarp (Figure 2).

Inheritance of targeted mutations and putative off-target analysis in T_1 generation

We further investigated six T_1 generation lines X134-#2, X134-#3, X134-#9, S143-#8, S143-#18 and S143-#32 for inheritance of the introduced mutations and segregation of the Cas9/sgRNA T-DNA. We observed that the mutations in the T_0 transgenic plants were stably transmitted to the T_1 generation (Table 2, Figure 3). Consistently, seeds of T_1 in-frame *Rc* mutant plants displayed the same red pericarp phenotype as those of T_0 plants. Our results also showed that the Cas9/sgRNA T-DNA could be segregated out in some T_1 homozygous mutant plants (Table 2, Figure 3).

The T_1 homozygous in-frame *Rc* plants were evaluated for potential off-target mutations. A total of 11 potential off-target sites (seven for sgRNA1 and four for sgRNA2, respectively) containing three to four mismatched bases were retrieved using the online tools CRISPR-P (http://skl.scau.edu.cn/offtarget/) and BLASTN (Tables 3 and S2). PCR products amplified from 10 T_1 plants (five of X134 lines and five of S143 lines) were sequenced, and no mutation events were detected in all potential off-target sites (Table 3), indicating that the two sgRNAs had high specificity in inducing mutagenesis of the *rc* allele.

Accumulation of proanthocyanidins and anthocyanins in the grains of in-frame *Rc* mutant plants

Seeds harvested from T_1 in-frame *Rc* lines X134-#2 (−15 bp/−15 bp), X134-#9 (−15 bp/−30 bp), S143-#18 (−15 bp/−20 bp) and S143-#32 (−18 bp/−18 bp) were subjected to measurement of oligomeric proanthocyanidin (OPC) content. We observed that OPC content in the seeds of in-frame *Rc* lines was over twofold and fivefold higher than that in the seeds of their corresponding wild types, X134 and S143, respectively (Figure 4a), confirming that red rice grains contain a high level of proanthocyanidins. Remarkably, there was no significant difference between OPC content in heterozygous plant S143-#18 (−15 bp/−20 bp) and that in homozygous plants S143-#32 (−18 bp/−18 bp; Figure 4a).

The X134-#2 (−15 bp/−15 bp) and X134-#9 (−15 bp/−30 bp) were further measured for the accumulation of four main types of anthocyanins: peonidin (PeoC), cyanidin (CC), delphindin (DC) and pelargonidin (PelC). While PeoC was not detected in any of the samples examined (data not shown), CC, PelC and DC in XS134-2 and XS134-9 grains were higher than that in wild-type seeds. The content of CC, which is considered to be the main anthocyanin component in red rice, was about 17-fold higher than that in the wild type (Figure 4b). The results also showed that restoration of *Rc* allele could result in accumulation of proanthocyanidins and anthocyanidins in rice grains, to levels comparable to or higher than that in a natural red rice variety.

Major agronomic and grain quality traits in T_1 generation

The transgene-free T_1 generation plants with homozygous in-frame *Rc* variants, X134-#2, X134-#9, S143-#18 and S143-#32, were cultured in the field and were investigated for major

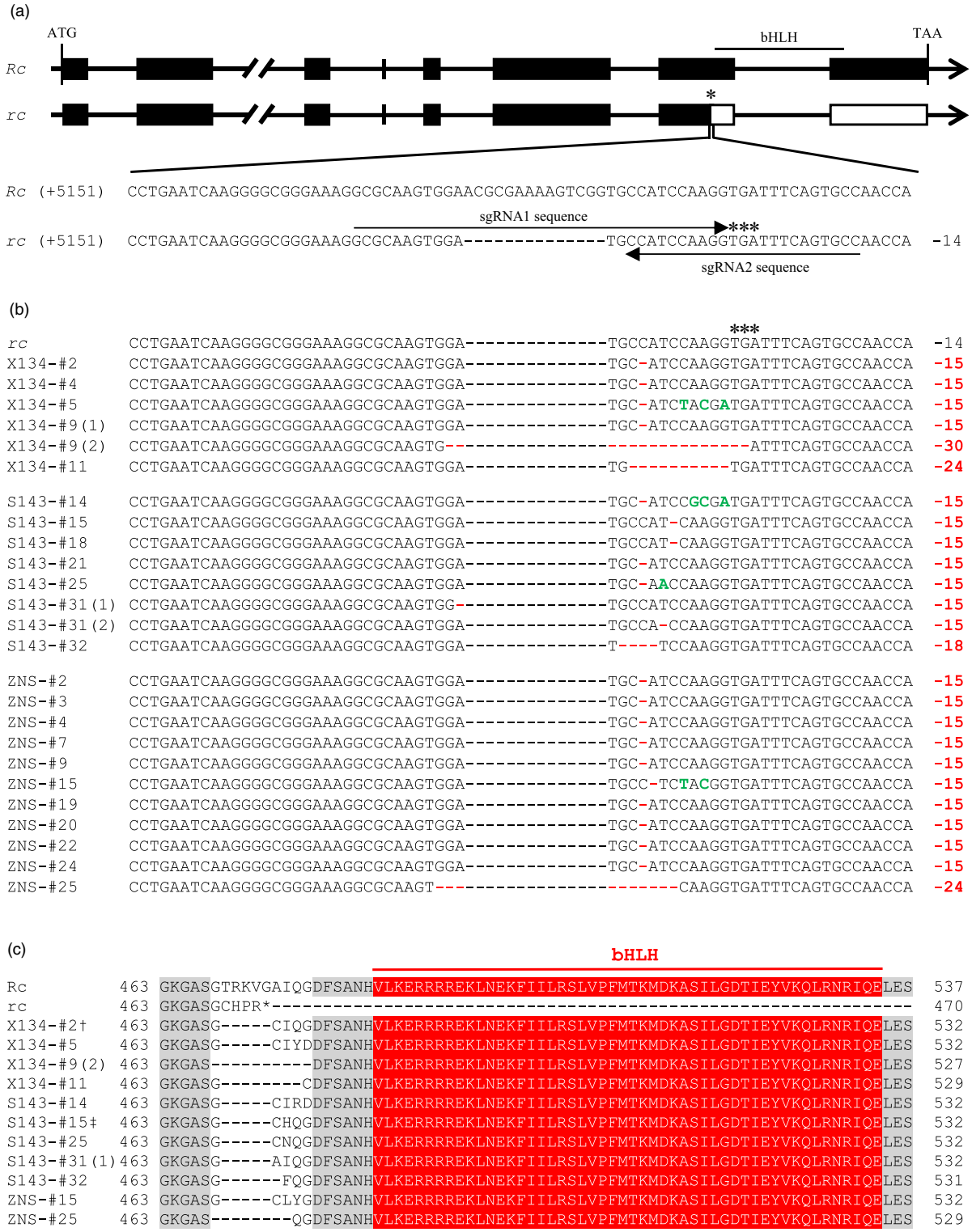

Figure 1 Development of elite red rice varieties through CRISPR/Cas9-mediated functional recovery of the recessive *rc* allele. (a) Gene structures of *Rc* in wild red rice (*Oryza rufipogon*) and *rc* in cultivated white pericarp rice. The 14-bp deletion and the premature stop codon in *rc* are indicated by black dashes and asterisks, respectively. The two sgRNAs are indicated by arrows. (b) Mutations adjacent to the 14-bp deletion site in T_0 plants harbouring in-frame *Rc* variants. Newly introduced deletions and substitutions are indicated by red dashes and green letters, respectively. Numbers on the right side indicate the lengths of deletions compared with wild-type *Rc* in *O. rufipogon*. (c) Multiple alignment of the deduced bHLH coding amino acid sequences of in-frame Rc variants. †X134-#2, X134-#4, X134-#9(1), S143-#21, ZNS-#2, ZNS-#3, ZNS-#4, ZNS-#7, ZNS-#9, ZNS-#19, ZNS-#20, ZNS-#22 and ZNS-#24 had an identical amino acid sequence. ‡S143-#15, S143-#18 and S143-#31(2) had an identical amino acid sequence.

Table 1 T_0 plants transformed with Cas9/sgRNA constructs targeting the flanking sequences of the *rc* 14-bp deletion site

Variety	No. of transgenic plants	No. of plants with mutations (%)†	T_0 zygosity‡			No. of plants with in-frame *Rc* alleles (%)‡
			Homozygous (%)	Bi-allelic (%)	Heterozygous (%)	
X134	12	9 (75.0)	2 (22.2)	7 (77.8)	0 (0.0)	5 (55.6)
S143	26	20 (76.9)	10 (50.0)	9 (45.0)	1 (5.0)	7 (35.0)
ZNS	22	17 (77.3)	9 (52.9)	8 (46.1)	0 (0.0)	11 (64.7)
Total	60	46 (76.7)	21 (45.6)	24 (52.2)	1 (2.2)	23 (50.0)

†Percentages were calculated over the total number of transgenic plants.

‡Percentages were calculated over the total number of plants with mutations.

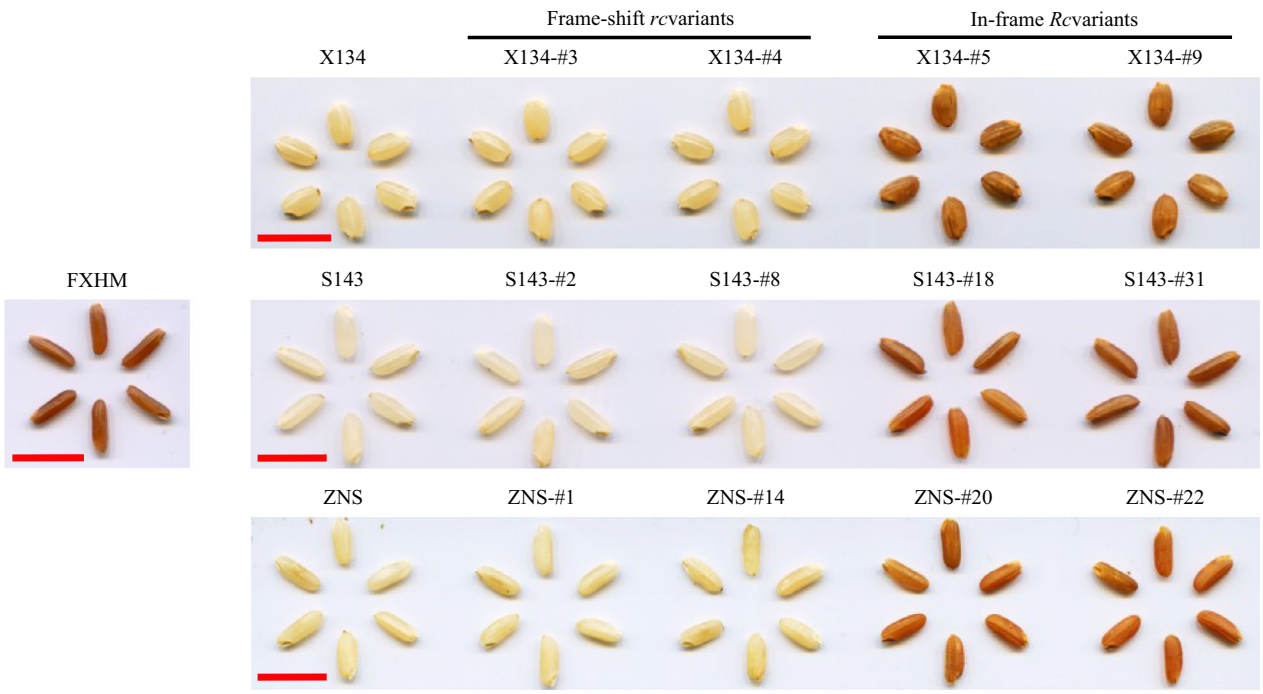

Figure 2 Pericarp phenotypes of T_0 mutant plants. FXHM was a commercial red rice variety used as a positive control. Seeds from ZNS mutants were produced by crossing with white rice restorer line MH86. Scale bar: 1 cm.

agronomic traits. The results showed that the in-frame *Rc* mutants did not display evident alterations in whole plant and seed morphology (Figure 5a–d). Further investigations on yield-associated traits showed that there were no significant differences in plant height, panicle number per plant, grain width, grain length, 1000-grain weight and grain yield per plant between the in-frame *Rc* mutants and their corresponding wild types X134 or S143, respectively (Figure 5e–f). The polished grains without red pericarp from T_1 homozygous in-frame *Rc* variants were also measured for major grain quality traits. The results showed that there were no significant differences in head rice rate, chalkiness rate, chalkiness degree, amylase content, gelatinization temperature of grains and gel consistency between the in-frame *Rc* mutants and their corresponding wild types X134 or S143, respectively (Figure S5). Taken together, these results demonstrated that restoration of *Rc* function had no negative effect on the important agronomic or grain quality traits in rice.

Discussion

Cultivated rice has long been domesticated from the wild rice to meet human needs (Fuller *et al.*, 2009; Huang *et al.*, 2012; Malory *et al.*, 2011). The rice seed is mainly made up of an outer husk layer, a pericarp layer, the starchy endosperm and the embryo. The change from red pericarp colour to white colour was an important hallmark of rice domestication (Sweeney *et al.*, 2007). It was speculated that ancient farmers selected and spread white rice varieties because white grains were easier to cook, easier to dehusk, or had better background for picking out diseases or insects (Sweeney *et al.*, 2007). Most cultivated rice varieties throughout the world today are white pericarp rice. In general, the husk and the pericarp layers are removed from the seeds of white varieties during the milling process, yielding polished white grains for consumption. In contrast, red rice varieties produce red pericarp covered with light-coloured husk and the seeds are milled only to remove the husk, but the

Table 2 Inheritance of the introduced mutations in six T_1 lines

		T_1 plant				
			No. of plants with mutation phenotype		*Hpt* and *Cas9* positive/*Hpt* and *Cas9* negative[§]	No. of transgene-free plants with homozygous in-frame *Rc* allele
T_0 plant	Mutation genotype of T_0 plant[†]	No. of plants tested	Bi-allelic	Homozygous[‡]		
X134-#2	−15 bp/−15 bp	8	0	8 (−15 bp)	5:3	3
X134-#3	−13 bp/−19 bp	9	4	3 (−19 bp); 2 (−13 bp)	7:2	0
X134-#9	−15 bp/−30 bp	24	13	6 (−30 bp); 5 (−15 bp)	17:7	3
S143-#8	−13 bp/−26 bp	15	9	4 (−26 bp); 2 (−13 bp)	10:5	0
S143-#18	−15 bp/−20 bp	16	7	4 (−20 bp); 5 (−15 bp)	12:4	2
S143-#32	−16 bp/−18 bp	24	13	6 (−18 bp); 5 (−16 bp)	18:6	2

[†]The numbers indicate the lengths of deletions compared with wild-type *Rc* in *Oryza rufipogon*; −: deletion; bi-allelic mutations are distinguished by '/'.

[‡]The numbers in brackets indicate the lengths of deletions compared with wild-type *Rc* in *O. rufipogon*.

[§]The presence/absence of *Hpt* and *Cas9* was determined by PCR amplification.

pericarp layer remains for consumption. The red pigment contained high levels of proanthocyanidins and anthocyanins, which have been shown to possess antioxidant properties against free radicals (Finocchiaro *et al.*, 2007; Gunaratne *et al.*, 2013; Qiu *et al.*, 2010). Due to its health-promoting benefits, red rice has become increasingly popular. In recent decades, although several red rice varieties have been developed based on conventional breeding (Sharma *et al.*, 2014; Zhang *et al.*, 2015), most cultivated red rice varieties suffer from low yield or other poor agronomic traits. Thus, development of elite varieties would be of great significance for production of red rice to meet the growing market.

The CRISPR/Cas9 system is a revolutionary approach that enables precise genome editing in various organisms. The novel biotechnological approach has been widely used in plant breeding for improving various traits through knocking out of the genes with deleterious on agronomic traits. In this study, we demonstrate that the CRISPR/Cas9 system could be an effective approach to restore recessive frame-shift alleles for crop improvement. We successfully converted three elite white pericarp rice varieties into red ones without compromising yield potential through CRISPR/Cas9-mediated functional recovery of the recessive *rc* allele. We generated a total of 60 T_0 CRISPR/Cas9 transgenic plants from three varieties and identified 46 (76.7%) plants with mutations in the target sites. Among the 46 plants, 23 (50%) contained at least one in-frame *Rc* variant and exhibited red pericarp. Given that about 97.9% of the white pericarp rice varieties are a result of the 14-bp deletion in *Rc* (Sweeney *et al.*, 2007), it is conceivable that our method could be applied to most white pericarp rice varieties. In addition, we observed no significant difference between wild-type and T_1 in-frame *Rc* plants in major agronomic or grain quality traits. We are now growing T_2 in-frame *Rc* lines under field conditions. Consistent with results observed in T_1 generation, there is no significant difference between wild-type and T_2 in-frame *Rc* lines in morphological traits (data not shown). Thus, our approach would greatly accelerate the breeding of new red rice varieties with elite agronomic traits.

The *Rc* gene has a dominant effect on the red coloration in rice grains (Furukawa *et al.*, 2007; Sweeney *et al.*, 2006). In the present study, we also observed that bi-allelic plants containing one dose of in-frame *Rc* exhibited red pericarp phenotype identical to that of homozygous in-frame *Rc* plants. Thus, it is feasible to develop hybrid red rice varieties through developing either red rice restorer lines or red rice sterile lines. In the present study, we successfully generated both the red rice restorer line and sterile line, allowing more flexibility for developing hybrid red rice combinations with elite agronomic traits.

Crop domestication is a process of artificially selecting ancestral wild plants and adapting them for human requirements: yield, taste, storage, etc. However, some beneficial or specialty traits may be lost during the long process. Most recently, genome editing technology has been demonstrated to speed up plant domestication. Editing of domestication-related genes in ancestral wild plants could mimic changes that occurred in plants during domestication. Using the CRISPR/Cas9 system, Lemmon *et al.* (2018) generated ground cherry (*Physalis pruinosa*) plants that yielded more and bigger fruit; Li *et al.* (2018) generated tomato plants having domesticated phenotypes from ancestral tomato strains with disease resistance and salt tolerance; and Zsögön *et al.* (2018) generated tomato plants with improved fruit size, fruit number and high accumulation of fruit lycopene from wild tomato (*Solanum pimpinellifolium*). In this study, we present an alternative strategy for editing of domestication-related genes for crop improvement. Our results demonstrated that editing of the recessive domestication-related *rc* gene allele could recover red pericarp, a specialty trait, in modern cultivated rice varieties. This strategy could be applied to develop new crop varieties with beneficial or specialty traits lost during the domestication process.

Experimental procedures

Plant materials and growth conditions

Three elite white pericarp rice varieties, including a *japonica* conventional variety Xiushui134 (X134), an *indica* restorer line Shuhui143 (S143) and an *indica* two-line sterile line ZhiNongS (ZNS), and a conventional red rice variety Fengxinhongmi (FXHM) were used in this study. Transgene-free plants were grown in a standard greenhouse and in the paddy field at Fuzhou experimental station (26.08°N, 119.28°E), Fujian Province, China. Transgenic rice plants were grown in a standard greenhouse.

Construction of CRISPR/Cas9 plant expression vectors and rice transformation

Two single-guide RNA (sgRNA) sequences targeting the flanking sequences of the 14-bp deletion site in the *rc* allele were

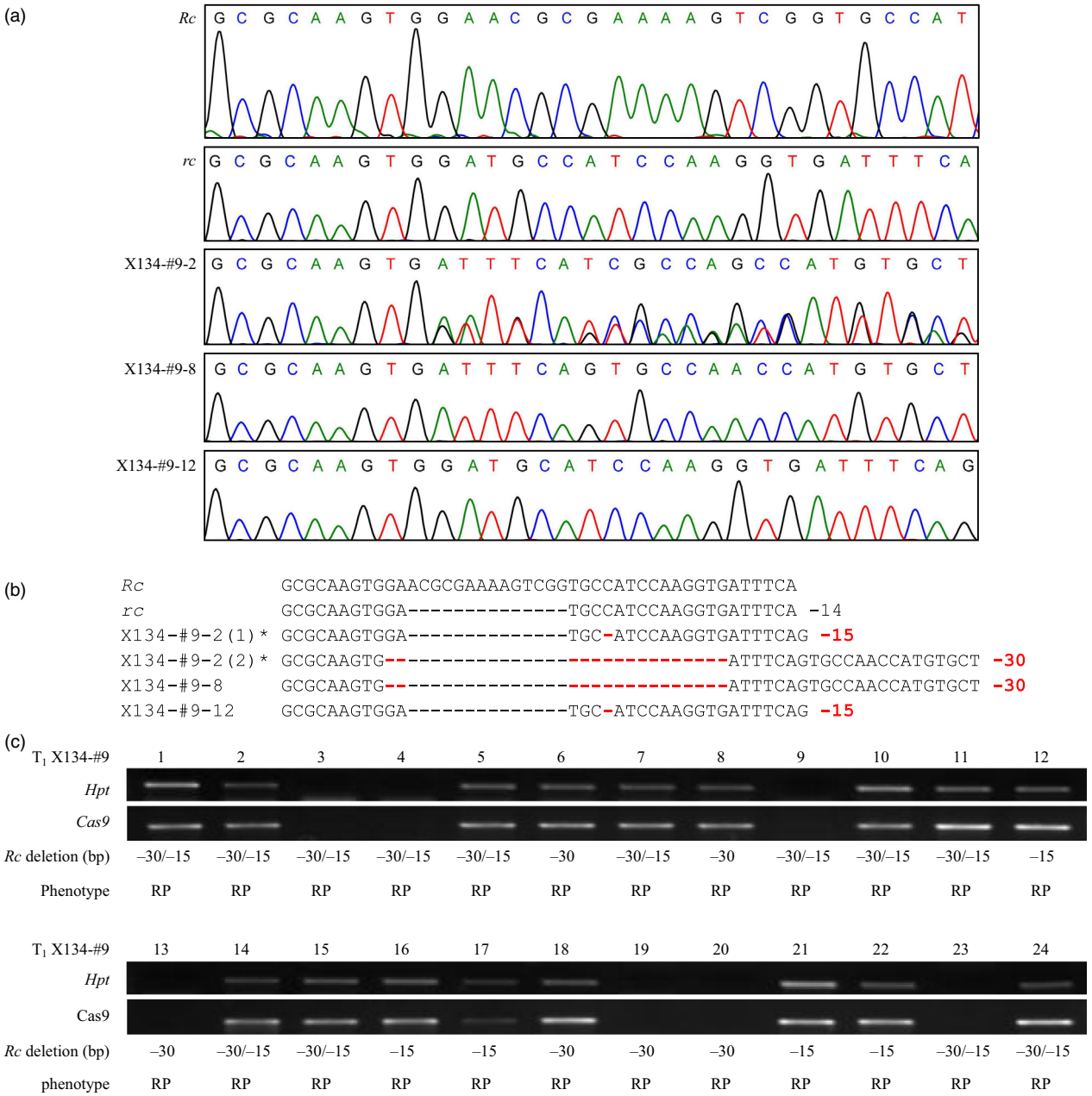

Figure 3 Segregation analysis of T_1 progeny of in-frame *Rc* line X134-#9. (a) Examples of sequencing chromatograms of the deletion site in the *Rc*/*rc* allele in wild red rice (*Rc*), white pericarp rice (*rc*) and T1 progeny of X134-#9. (b) Alignment of sequences decoded from sequencing chromatograms of the deletion site in the *Rc*/*rc* allele in wild red rice (*Rc*), white pericarp rice (*rc*) and T1 progeny of X134-#9. *Bi-allelic sequences were decoded from sequencing chromatograms using an online program DSDecode (http://dsdecode.scgene.com/). (c) Segregations of the *Rc* deletion genotypes and the Cas9/sgRNA T-DNA in T1 progeny of X134-#9. The presence/absence of the Cas9/sgRNA T-DNA was determined by PCR amplification for the *hygromycin phosphotransferase* (*Hpt*) gene and the Cas9 gene. RP, red pericarp.

designed as predicted by the CRISPR-P program (Lei *et al.*, 2014). The oligonucleotides corresponding to the designed sgRNA sequences were synthesized, and oligonucleotide dimers were cloned into a CRISPR/Cas9 plant expression vector VK005-01 (ViewSolid Biotech, Beijing, China) according to the manufacturer's instruction. The resulting constructs contained a *Cas9* gene driven by the maize ubiquitin promoter and a designed sgRNA sequence under control of the rice *U6* promoter.

The Cas9/sgRNA constructs were transfected into Agrobacterium tumefaciens EHA105 by electroporation. Rice calli of X134, S143 and ZNS were transformed with the mixture of *Agrobacterium* strains harbouring the Cas9/sgRNA constructs. Generation of transgenic rice plants was carried out as previously described (Hiei *et al.*, 1994).

Genotype and agronomic trait analysis

Genomic DNAs were extracted from rice leaves using the hexadecyltrimethylammonium bromide (CTAB) method (Porebski *et al.*, 1997). PCR amplifications were carried out to genotype the seventh exon of the *Rc*/*rc* allele, the coding exons of the *Rd* gene

Table 3 Evaluation of potential off-target sites

Target	Name of putative off-target sites	Putative off-target locus	Putative off-target sequence	No. of mismatch bases	No. of plants examined	No. of indel mutation
sgRNA1	1-OFF1	ch02: 28556203–28556225	GCTCAAGTCGACGCCCTCCAAGG	4	10	0
	1-OFF2	ch03: 21512179–21512201	CGGCAAGTGGATGCCATCTATGG	3	10	0
	1-OFF3	ch04: 26251953–26251975	GCGCAAGTGGATGGTTTCCTGGG	4	10	0
	1-OFF4	ch08: 25739010–25739032	GCTGAGGTGGAGGCCATCCAAGG	4	10	0
	1-OFF5	ch09: 8657855–8657877	CGGCAAGTGGATGCCATCTATGG	3	10	0
	1-OFF6	ch10: 10111987–10112009	GGGCAAATGGATGGCATGCAAGG	4	10	0
	1-OFF7	ch11: 17602327–17602349	GCGCGACTGGATGGCATCCATGG	3	10	0
sgRNA2	2-OFF1	ch05: 13717502–13717524	GGCTCTGAAGTCCCTTTGATGG	4	10	0
	2-OFF2	ch05: 20480244–20480266	GGCTCTGAAGTCCCTTTGATGG	4	10	0
	2-OFF3	ch09: 20085301–20085323	GGCACTGAAAATTCCTCGGATGG	4	10	0
	2-OFF4	ch12: 15994192–15994214	GGCACCGAAATAATTTTGGATGG	4	10	0

PAM sequence NGG is indicated in blue. Mismatch nucleotides are marked in red.

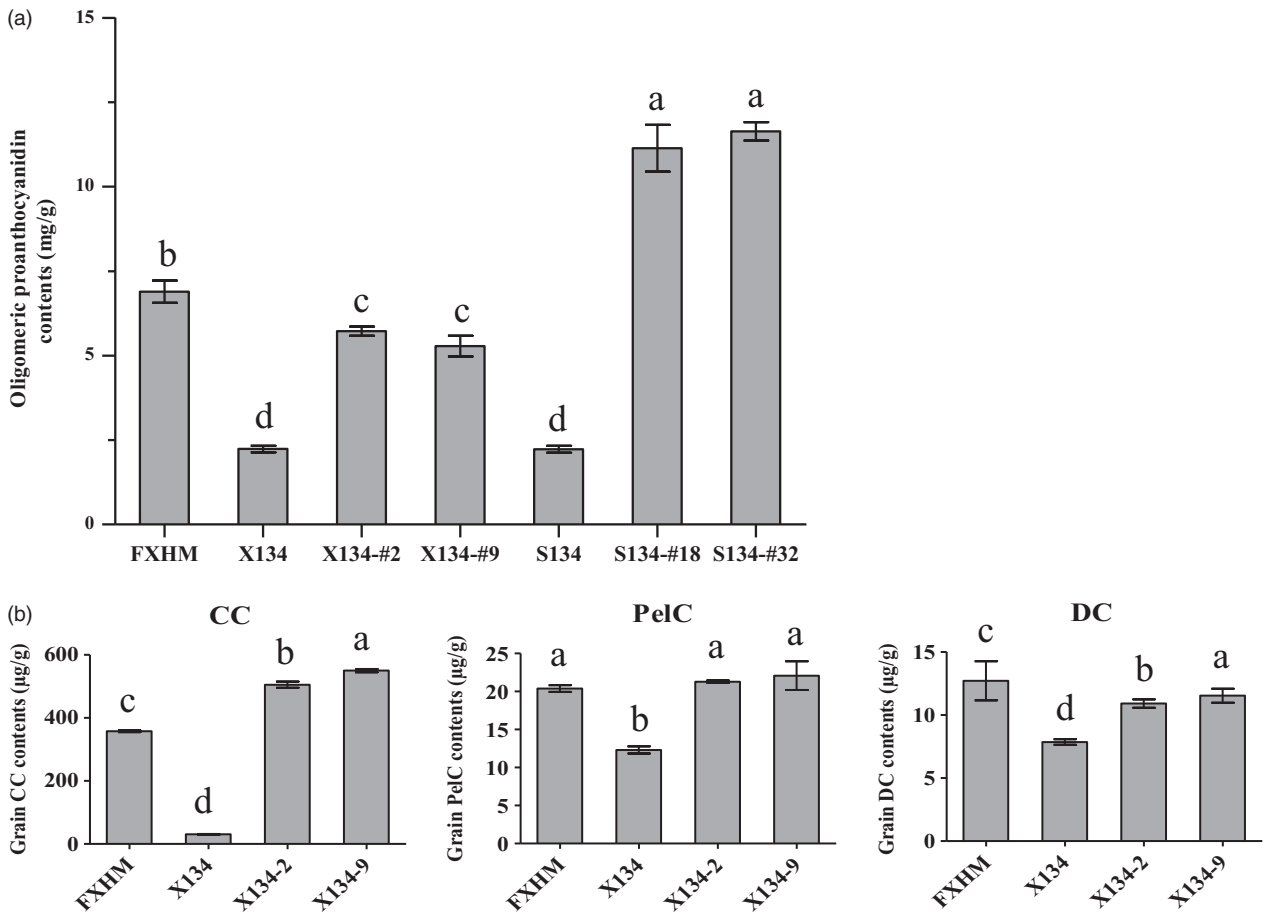

Figure 4 Oligomeric proanthocyanidin and anthocyanidin contents in grains of red rice lines. (a) Oligomeric proanthocyanidin contents in grains of red rice lines in X134 and S143 backgrounds. FXHM was a commercial red rice variety used as a positive control. Data are presented as mean ± SD. Different letters indicate groups with significant differences (ANOVA, $P < 0.05$). (b) Grain CC, PelC, DC contents (μg/g) of red rice lines in XS134 and corresponding their control lines. Data are presented as mean ± SD. Different letters indicate groups with significant differences (ANOVA, $P < 0.05$).

in X134, S143 and ZNS, and mutations in the *rc* allele in transgenic plants. The PCR products were sequenced by Sanger method. Decoding of sequencing chromatograms was carried out as described (Liu *et al.*, 2015). DNA sequences were aligned using Clustal Omega (Sievers *et al.*, 2011). Segregation of the Cas9/sgRNA T-DNA was investigated in T_1 generation derived from self-pollination of T_0 mutant plants. The presence/absence of the Cas9/sgRNA T-DNA was evaluated by PCR amplification using specific primers for the *hygromycin phosphotransferase* (*Hpt*) gene and the *Cas9* gene.

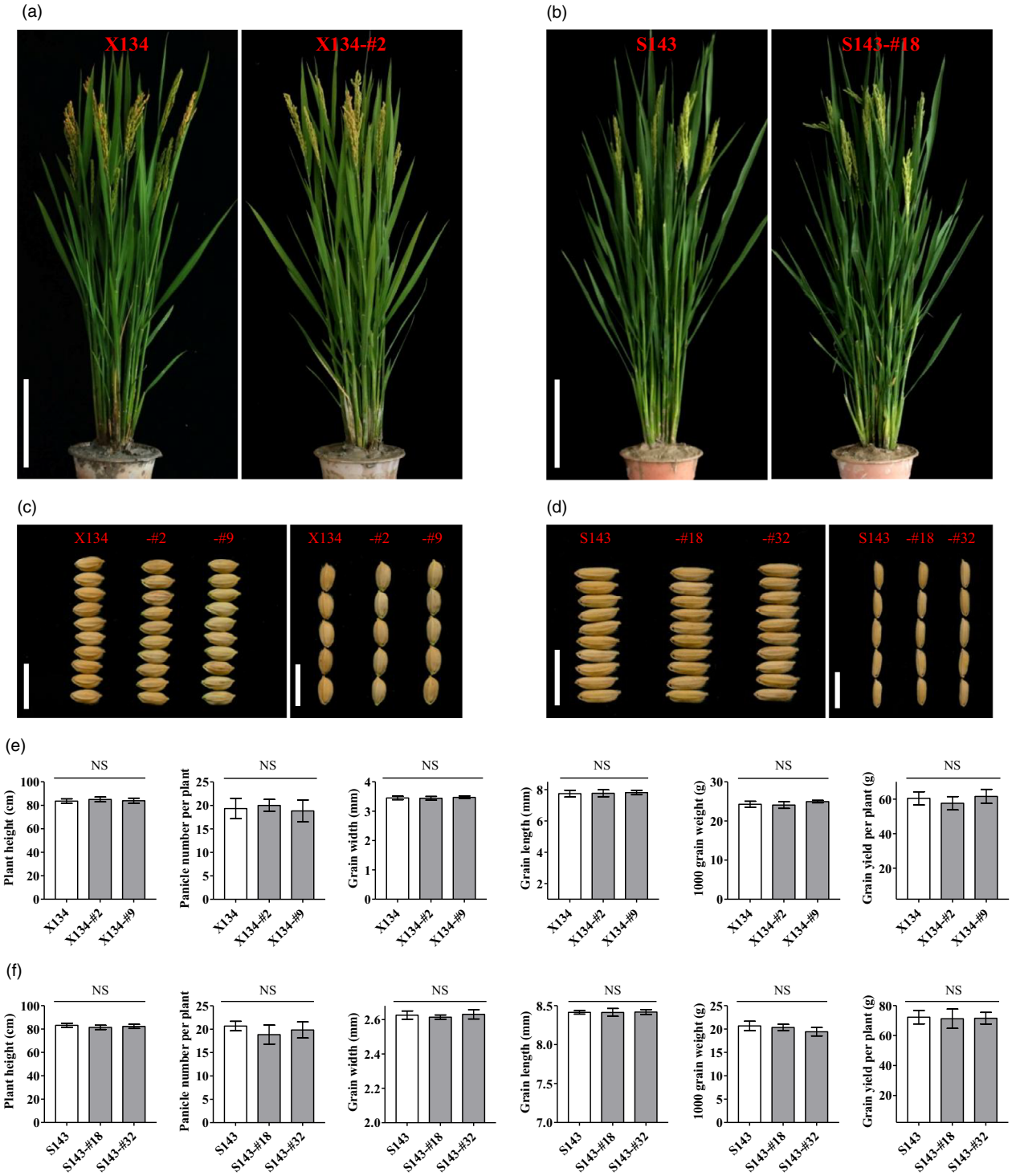

Figure 5 T_1 generation homozygous in-frame *Rc* variants and their corresponding wild types showed no significant differences in major agronomic traits. (a, b) Morphological phenotypes of in-frame *Rc* mutants and their corresponding wild types X134 and S143. Scale bar: 20 cm. (c, d) Phenotypes of seeds with husk of in-frame *Rc* mutants and their corresponding wild types X134 and S143. Scale bar: 1 cm. (e, f) Plant height, panicle number per plant, grain width, grain length, 1000-grain weight and grain yield per plant in in-frame *Rc* mutants and their corresponding wild types X134 and S143. NS, no significant difference.

Wild-type parents and T_1 generation plants were investigated for agronomic traits. Rice plants were cultivated at Fuzhou experimental station, Fujian Province, under nature long-day condition (mid-May to mid-September, c. 13.5 h). The management of plants was followed the normal agricultural practice.

All primers used for in this study are listed in Table S2.

Measurement of oligomeric proanthocyanidins and anthocyanins

One gram of rice seeds was de-hulled and ground enough to pass through a 40-mesh sieve. The content of oligomeric proanthocyanidins (OPCs) in rice seeds was determined with a Micro Plant Proanthocyanidins Assay Kit (Solarbio Science & Technology, Beijing, China) following the manufacturer's instructions.

Approximately 2 g of rice seeds was powdered after freezing in liquid nitrogen and was homogenized in methanol/hydrochloric acid extraction buffer by sonication according to the method described previously (Abdel-Aal *et al.*, 2006; Furukawa *et al.*, 2007). The extract was centrifuged at 12 000 ***g*** for 10 min. The supernatant was treated with concentrated hydrochloric acid and incubated at 95 °C for 40 min. After cooling, the crude extract containing the anthocyanins was passed through a 0.22-μm filter and quantified by accurate mass HPLC1290/MS-Qtrap 6500 (Agilent Technologies, Palo Alto, CA) system packed with a reversed-phase column (2.1 × 150 mm, 2.7 μm).

Measurements of OPCs and anthocyanins were based on at least three replicates. Data are presented as means ± SD. The statistical significance of the differences in the contents was determined by ANOVA.

Grain quality trait analysis

Polished grains without pericarp were measured for major grain quality traits. The head rice rate, chalkiness rate and chalkiness degree were measured by a rice-grain appearance analysis system (Wseen, China). The amylose content was obtained by following the ISO-6647 method. The gelatinization temperature was determined using the alkali digestion test (Little *et al.*, 1958), and the gel consistency of grain was measured as previously described (Cagampang *et al.*, 1973).

Acknowledgements

This work was supported by Science and Technology Major Project of Fujian Province (2015NZ0002-3), National Key Research and Development Program of China (2016YFD0100903) and Fujian Provincial Special Fund (2015R1019-1).

Conflicts of interest

The authors declare that they have no conflicts of interest with respect to this work.

Author contributions

F.W., Y.Z. and L.C. conceived the project and designed the experiments. Y.Z., Y.L., S.C., M.F., Z.C., T.H., H.L., F.M. and J.C. performed the experiments. Y.Z, Y.L. and S.C. analysed the data. Y.Z., Y.L. and S.C. wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Multiple alignment of the seventh exon of the *Rc*/*rc* allele in wild red rice (*Oryza rufipogon*), and three white pericarp rice varieties X134, S143, and ZNS.

Figure S2 Multiple alignment of the coding regions of the *Rd* gene in wild red rice (*Oryza rufipogon*), and three white pericarp rice varieties X134, S143, and ZNS.

Figure S3 Multiple alignment of the deduced amino acid sequences of in-frame *Rc* variants.

Figure S4 In-fame *Rc* mutants in ZNS background displayed an identical male sterility phenotype as their wild type.

Figure S5 In-fame *Rc* mutants and their corresponding wild types showed no significant differences in major grain quality traits.

Table S1 Genotype of T_0 mutant plants in three rice varieties.

Table S2 Primers used in this study.

