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Article

Removing Harmful Pericarp Character of Weedy Rice as the First Step of Domestication towards Direct-Seeding Rice Using CRISPR/Cas9-Targeted Mutagenesis

Mengyao Kong †, Xiaotong He †, Zhendong Yin, Xianshu Chen, Yujie Zhang, Zhihua Shi, Xiaoling Song, Sheng Qiang and Weimin Dai *

Weed Research Laboratory, Department of Plant Science, College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China
* Correspondence: daiweimin4@njau.edu.cn
† These authors contributed equally to this work.

Abstract: With the scarcity of fresh water resources and the increase in labor prices, rice cultivation is changing from transplanting to direct seeding. Weedy rice is a malignant weed with strong drought tolerance in seed germination in direct-seeding rice fields. As the same species of cultivated rice (*Oryza sativa* L.), weedy rice (*Oryza sativa* f. *spontanea*) has the potential of domestication into direct-seeding rice with strong drought tolerance in seed germination by changing a few unfavorable traits. The red pericarp, one of the harmful traits of weedy rice, seriously affects the quality and commercial value of cultivated rice. The recently developed CRISPR/Cas9 (Clustered Regular Interspaced Short Palindromic Repeats) technology can accurately edit the function of genes, providing a possibility for the directed evolution. Weedy rice (WRL-162) with red pericarp was used as a mutant material. Two mutation sites, upstream and downstream of the 14 bp differential fragment of exon 6 of *Rc* gene, were designed using the CRISPR-Cas9 gene-editing technique. Consequently, three mutant types in T1 generation weedy rice with CRISPR-Cas9 vector changed from red to white pericarp. One mutant type in T1 generation weedy rice with CRISPR-Cas9 vector maintained red pericarp. Comparing the wild type of weedy rice and rice variety control (Nipponbare), mutant types in T3 generation weedy rice without CRISPR-Cas9 vector significantly improved the drought resistance in seed germination. In addition, there was no significant difference in panicle number, seed setting rate per plant, grain length and width between wild type and T3 generation mutant weedy rice without CRISPR-Cas9 vector. Comparing the wild type, some mutant types in T3 generation weedy rice without CRISPR-Cas9 vector significantly decreased plant height, decreased spikelet number per main panicle, decreased plump seed number per main panicle, and increased 1000-grain weight. Our results showed that the *Rc* gene editing using CRISPR/Cas9 technology can not only remove the harmful pericarp character of weedy rice, but also improve drought tolerance in seed germination. This study might provide new insights for the utilization of weedy rice as germplasm resources for direct-seeding rice by precisely editing genes to remove unfavorable traits.

Keywords: weedy rice; germplasm resources; direct-seeding rice; CRISPR/Cas9; pericarp color; *Rc* gene

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1. Introduction

Weedy rice, also known as red rice because of its usually red pericarp, could naturally reproduce in rice fields and occur in most rice-growing areas around the world [1]. Weedy rice is highly competitive and can significantly reduce crop yields and quality. The annual economic loss in the United States is expected to exceed USD 45 million [2,3]. In the past few decades, with the increase in water resources and labor prices, rice production from direct seeding instead of transplanting has been believed to promote the global issues due to weedy rice [4–6].

Weedy rice is de-domesticated from cultivated rice [7–11] and has an abundant genetic variation [1,12,13]. The plant architecture and seed shattering of some weedy rice accessions are very similar to coexisting cultivated rice [1,14]. During the de-domestication process of weedy rice, new mutations were generated to adapt to the local environment [9]. Some weedy rice accessions have tremendous tolerance to drought, aging, salt and alkali, cold, various diseases, and insects [6,11,15–17]. Wang et al. [9] found that weedy rice has a stronger seed germination ability than the coexisting cultivated rice in 20% Polyethylene glycol-6000 (PEG-6000) solution, which might partly explain the cause of the especially serious damage of weedy rice in a direct-seeding rice field. Therefore, weedy rice could be domesticated as a germplasm resource of direct-seeding rice with strong competitiveness such as drought tolerance in seed germination by precisely removing a few unfavorable traits.

Mixing weedy rice with red pericarp into cultivated rice with white pericarp could seriously affect the quality and commercial value of cultivated rice [1]. The dominant *Rc* gene plays a key role in the formation of red pericarp in rice [18]. *Rc* gene is located on chromosome 7 and measures approximately 6400 bp in length [7]. The *Rc* gene encodes a basic helix–loop–helix (bHLH) protein that contains four conserved functional regions, namely the interaction region (I), the acidic region (A), the basic helix–loop–helix region (bHLH), and the C-terminal region (C) [19–21]. The recessive gene (*rc*) lacks 14 bp in its exon 6 compared to the dominant *Rc* gene [7,8]. The American cultivar "Wells" and Italian variety "Perla" produced a red pericarp mutant through the deletion of natural mutations, thereby restoring the bHLH protein structure of the *Rc* gene [22,23]. Therefore, it is possible to disable the function of *Rc* by precisely mutating the *Rc* gene and change the pericarp color of the weedy rice from red to white.

The *Streptococcus pyogenes* CRISPR-Cas9 system has been used for genome editing in a variety of organisms. It generates double-strand breaks (DSBs) at specific sites in chromosomal DNA and introduces INDEL mutations at DSB [24–27]. Zhu et al. (2019) [28] reported that the white pericarp cultivated rice could be mutated to red pericarp cultivated rice using the CRISPR/Cas9 technique on the *rc* gene. However, could red pericarp be mutated to white pericarp using the CRISPR/Cas9 technique? Would the weedy rice with the *Rc* gene edited decrease or increase drought tolerance in seed germination? Would the weedy rice with the *Rc* gene edited change other morphological traits? The study answered the three questions above and further provided new insights for the utilization of weedy rice as a rice germplasm resource.

2. Materials and Methods

2.1. Materials

The weedy rice accession (Japonica type, Field No. 162) was collected from Jiangsu Province of the Weed Research Laboratory of Nanjing Agricultural University and bagged to purify for 10 generations. The *Rc* gene sequences of weedy rice (Field No. 162) were deposited in NCBI website (GenBank number: MK797991). pRGEB32 was used for the CRISPR/Cas9 vector [24]. The primers used in this research were synthesized by Nanjing Jinsirui Biotechnology Co., Ltd. (Nanjing, China), and the sequencing work was carried out by Shanghai Shenggong Biological Co., Ltd (Shanghai, China). (Supplementary Table S1).

2.2. Construction of CRISPR/Cas9-Rc Mutant Vector

The gRNA spacer (Supplementary Table S1) of the *Rc* gene was designed using the CRISPR PLANT website (accessed on 15 June 2018; http://www.genome.arizona.edu/crispr). The structural and functional regions of the *Rc* protein were analyzed with NCBI (accessed on 15 June 2018; https://www.ncbi.nlm.nih.gov/) and MSU (accessed on 15 June 2018; http://rice.plantbiology.msu.edu/).

According to the 14 bp difference between *Rc* and *rc* alleles [29], two pairs of primers (*Rc* 1-Forward, *Rc* 1-Reverse, *Rc* 2-Forward, and *Rc* 2-Reverse) were designed and synthesized separately. The two pairs of primers were annealed to form gRNA spacer *Rc*

1 and gRNA spacer *Rc* 2, respectively (Supplementary Table S1). The reaction mixture included forward primer 1 µL (100 µM), reverse primer 1 µL (100 µM), 10 × T4 DNA ligase buffer 1 µL (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, pH 7.5 (NEB company (Ipswich, MA, USA) and 7 µL ddH_2O, and placed in a PCR (Takara Company, Kusatsu, Japan). The PCR program was 37 °C for 60 min, 95 °C for 10 min, and 25 °C for 1 min. The expression vector pRGEB32 was digested with restriction endonuclease *BsaI* and separated on 1% agarose gel (electrophoresis at 140 V for 15 min). The 15 kb band of the expression vector pRGEB32 was cut from the agarose gel and purified using a kit (MiniBEST Agarose Gel DNA Extraction Kit Ver. 4.0; Takara company). The purified DNA was ligated with the above gRNA spacer (40-fold dilution of the 100 µM double-stranded product) at a 1:1 volume ratio with T4 ligase in a 4 °C refrigerator overnight. Subsequently, the ligation solution was transferred to competent *E. coli* DH5α and cultured in an LB medium containing kanamycin (50 mg L^{-1}).

Twelve positive monoclonal clones were picked and amplified with PCR using pRGEB32 vector primers (pRGEB32-3-F and pRGEB32-3-R) (Supplementary Table S1). PCR was performed with 4.0 µL 2 × PCR Mix, 4.5 µL ddH_2O, 0.25 µL pRGEB3-3-F (10 µmol L^{-1}), 0.25 µL pRGEB32-3-R (10 µmol L^{-1}), and 1 µL DNA (10–20 ng μL^{-1}). PCR Mix (Nanjing Jitian Biotechnology Co., Ltd., Nanjing, China) composed of dNTP 0.2 mmol L^{-1}, KCl 100 mmol L^{-1}, Tris-HCl (pH 8.5) 20 mmol L^{-1}, Taq Polymerase 5 U/100 µL, and $dMgCl_2$ 3.0 mmol L^{-1}. The PCR was set to provide the initial denaturation at 94 °C for 5 min, then 30 cycles (94 °C 45 s, 55 °C 45 s, 72 °C 1 min), and finally extended at 72 °C for 8 min. The PCR product was sent to Shanghai Shenggong Biological Co., Ltd. for the Sanger sequencing and verification the *Rc* gene mutation vectors pRGEB32-*Rc* 1 and pRGEB32-*Rc* 2 [24] (Supplementary Table S1).

2.3. Agrobacterium-Mediated Genetic Transformation of Weedy Rice

The constructed plasmid vectors (pRGEB32-Rc 1 and pRGEB32-Rc 2) were separately transferred to *A. tumefaciens* EHA105 (Biomed company) and stored at –80 °C for genetic transformation [30]. After disinfecting with 75% ethanol and 30% sodium hypochlorite, the caryopsis was inoculated on NB (Nutrient Broth) medium containing 2,4-D hormone. After two weeks of dark culture, a subculture was carried out. A friable callus was selected for the *Agrobacterium* infection. After the infection with *Agrobacterium*, the callus was incubated in the dark for two days. Then, the callus was subcultured twice in the hygromycin containing medium for the selection with the incubation period of 15 days each. The resistant callus was obtained after one month and transferred to the differentiation medium at 28 °C under light irradiation [30].

2.4. Molecular Identification of Rc Mutants

Genomic DNA was extracted from seedling leaves using Plant Genomic DNA Kit (TIANGEN company (Beijing, China). Hygromycin-positive T1 generation plants with CRISPR/Cas9 vector were screened with the specific primers of hygromycin gene, Cas9 gene, and UBI promoter (Supplementary Table S1). T2 and T3 generation mutant plants without CRISPR/Cas9 vector were also screened with the specific primers of hygromycin gene, Cas9 gene, and UBI promoter (Supplementary Table S1).

Furthermore, the *Rc* target site was separately amplified by specific primers (*Rc*-Test-5F, *Rc*-Test-5R, and *Rc*-Test-6F and *Rc*-Test-6R) and identified through Sanger sequencing. For plants sequenced to be bimodal, it is presumed to be a biallelic or heterozygous mutant. The PCR products were purified using a kit (MiniBEST Agarose Gel DNA Extraction Kit Ver. 4.0; Takara company) and ligated into the pMD19-T vector (Takara company). The ligation system of pMD19-T vector included 4.5 µL purified PCR product, 0.5 µL pMD19-T plasmid and 5 µL ddH_2O. For the ligation, the mixture was placed in a PCR at 16 °C overnight. The ligation product (10 µL) was introduced into competent DH5α (Takara company; 50 µL) using a heat shock method. Blue–white screening (X-gal 40 mg L^{-1}, IPTG 24 mg L^{-1}) was performed in an LB medium containing ampicillin (100 mg L^{-1}). Finally, six white DH5α

clones were picked to be amplified with universal primers, M13 (-47) and M13 (-48), and verified using Sanger sequencing (Supplementary Table S1).

2.5. Phenotype Traits

T1–T3 generation plantlets were transplanted into plastic buckets with a diameter of 26 cm and a depth of 22 cm. Then, the plastic buckets were placed in a greenhouse and normal agronomic management was applied, including watering, manure and pest control. The experiment was conducted in the greenhouse (32°1′7.4208′ N, 118°51′26.392′ E) of Nanjing Agricultural University in Nanjing city, Jiangsu Province, Southern China.

A total of 50 days after heading, the following traits were measured, including plant height, panicle number, spikelet number in main panicle, plump grain number in main panicle, seed setting rate per plant and 1000-grain weight. There was a total of 12 plants per replicate for three replicates [31,32]. Using a Vernier caliper (200 mm, 8′′, Xifeng, B-floor-145, Xinfa Economic City Business Building, Songjiang District, Shanghai), grain length and width were measured in 3 replicates with 12 seeds per replicate. With the help of an anatomical lens (10 × 0.8 times; Olympus-SZX7; Olympus Co. (Tokyo, Japan), the phenotypes of the hull color and pericarp color were recorded.

2.6. Drought Tolerance in Seed Germination

Previous studies have shown that weedy rice and cultivated rice seeds have weak dormancy [17,33]. Rice seed dormancy can be broken in a 45 °C oven for 48 h [31,32]. PEG-6000 is a macromolecule functioning as an osmotic regulator without damaging cells; 20% PEG-6000 solution is often used to simulate drought stress [34,35]. Four treatments were designed for seed germination experiments, namely distilled water germination, 20% PEG-6000 drought tolerant germination, distilled water germination after weak dormancy breaking, and 20%PEG-6000 drought tolerant germination after weak dormancy breaking.

Plump seeds were placed on two layers of filter paper in Petri dishes. Seeds were germinated in four treatments, including with 6 mL distilled water, with 6 mL distilled water and seeds pretreated in a 45 °C oven for 48 h, with 6 mL 20% PEG-6000 solution, with 6 mL 20% PEG-6000 solution and seeds pretreated in a 45 °C oven for 48 h, respectively. Each treatment was repeated three times with 30 plump seeds per replicate. The Petri dishes were wrapped in plastic bags and placed into incubators (SPX-400-GBH) at a steady temperature of 25 °C with a light–dark cycle of 16:8 h. The number of germinated seeds was recorded each day over the next 7 days. Germination was evaluated visually through the protrusion of the radicle from the hull by ≥3 mm [17,36].

2.7. Data Analysis

Sequence alignment and analysis of DNA sequencing fragments were performed using DNAman [37]. The sequence of the *Rc* gene was translated into amino acids by DNASTAR [38] to find out the mutation target site and bHLH region. One-way analysis of variance (ANOVA) was performed with SPSS 18.0 (Statistical Product and Service Solutions, SPSS, Chicago, IL, USA). For mapping, Origin 8 (Originlab, Northampton, MA, USA) was used.

3. Results

3.1. Construction of CRISPR/Cas9-Rc Mutant Vector and Agrobacterium-Mediated Genetic Transformation

In order to construct pRGEB32-*Rc* 1 and pRGEB32-*Rc* 2 vector, gRNA spacer-*Rc* 1 and gRNA spacer-*Rc* 2 were ligated to pRGEB32, respectively. PCR amplification was carried out using the vector detection primers (pRGEB32-3-F and pRGEB32-3-R) (Supplementary Figure S1; Supplementary Table S1) followed by sequencing (Supplementary Figure S2). The constructed mutant vectors (pRGEB32-*Rc* 1 and pRGEB32-*Rc* 2) were individually transferred to *A. tumefaciens* EHA105 and regenerated plantlets were obtained (Supplementary Figure S3).

3.2. Molecular Identification of T1-T3 Generation Plantlets

The hygromycin gene, Cas9 gene, and UBI promoter on the CRISPR/Cas9-*Rc* mutant vector were detected using PCR amplification in T1 generation plants, respectively (Figure 1; Supplementary Table S1). The *Rc* gene of positive plantlets with vector were sequenced to verify the mutation site of *Rc* gene (Table 1).

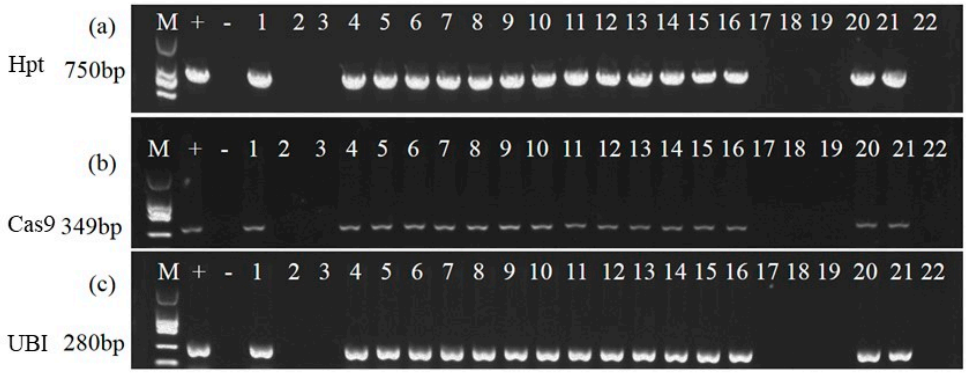

Figure 1. Molecular identification of T1 plantlets for weedy rice. Note: Hpt (Hygromycin gene), Cas9 (Cas9 gene) and UBI (promotor sequence) are all located on the pREGB32 vector. In (**a**–**c**), Hpt, Cas9 and UBI was detectd respectively. M was Marker. "+" was pREGB32 vector as positive control. "−" was water as negative control. 1–22 were T1 generation plants, respectively. Primers in Supplementary Table S1.

Table 1. Pericarp color and *Rc* genotype of T1–T3 generation plantlets for weedy rice (Field No. 162).

Positive Regenerated Plants	Mutation Type		Mutation Sequence	Phenotype of Pericarp Color
Wrl-162	Wild type		ACCTGAATCAAGGGGCGGGAAAGGCGCAAG TGGAACGCGAAAAGTCGGTGCCATCCAAG	Red
Rc 1-162-18	−6 bp	−6 bp	ACCTGAATCAAGGGGCGGGAA——AAGT GGAACGCGAAAAGTCGGTGCCATCCAAG	Red
Rc 1-162-1	−13 bp	−13 bp	ACCTGAATCAAGGGGCGGGAAAGG CG————-AAAAGTCGGTGC CATCCAAGGTGATTTCA	White
Rc 2-162-16	+T	+1 bp	ACCTGAATCAAGGGGCGGGAAAGGCGCTAAG TGGAACGCGAAAAGTCGGTGCCATCCAAG	White
Rc 2-162-17	+A	+1 bp	ACGCGAAAAGTCGGTGCCATCCAAGGTGA TTTCAGTGCCAACCATGTGCTGAAAAGAG	White

Note: The green base is the target site sequence of CRISPR/cas9. The blue base is the 14 base difference between *Rc* and *rc* allele. The red base is the base inserted after the mutation, and the red "−" sign is the deleted base (the number of "−" is the same as the number of the deleted base). *Rc* 1 and *Rc* 2 represent regenerated plants from the *Rc* gene knockout vectors pRGEB32-*Rc* 1 and pRGEB32-*Rc* 2, respectively.

Among the T1 generation plantlets of weedy rice (Figure 2; Table 1), *Rc*1-162-18 possessed a 6-base deletion mutation with a red pericarp, *Rc*1-162-1 possessed a 13-base deletion mutation with a white pericarp, *Rc*1-162-16 contained a mutation inserted into a single base "T" with a white pericarp, and *Rc*2-162-17 contained a mutation inserted into a single base "A" with a white pericarp (Table 1).

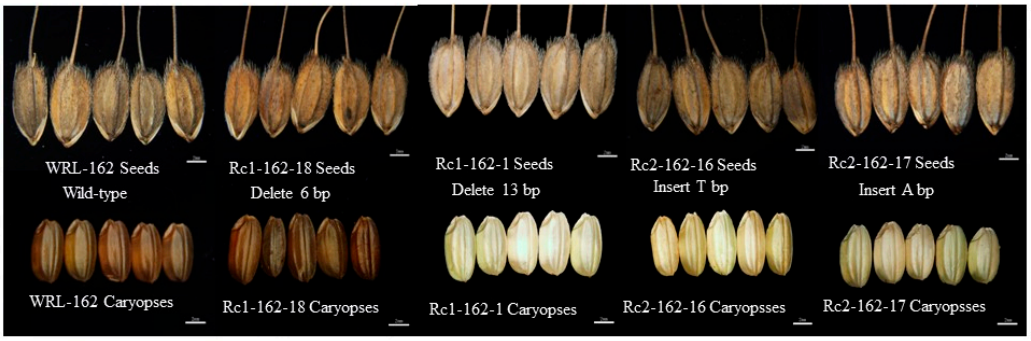

Figure 2. Seeds and caryopses of wild type and T3 mutants. Note: Wild type is Wrl-162. Mutants are *Rc*1-162-18, *Rc*1-162-1, *Rc*1-162-16, and *Rc*1-162-17. *Rc* 1 and *Rc* 2 represent regenerated plants from the *Rc* gene knockout vectors pRGEB32-*Rc* 1 and pRGEB32-*Rc* 2, respectively.

Among the T2 and T3 generation plantlets of weedy rice (Figure 3; Table 1), the hygromycin gene, Cas9 gene, and UBI promoter on the CRISPR/Cas9-*Rc* mutant vector were detected using PCR amplification (Figure 3; Supplementary Table S1). The vector free plantlets continued to be sequenced to verify the mutation site of *Rc* gene (Table 1).

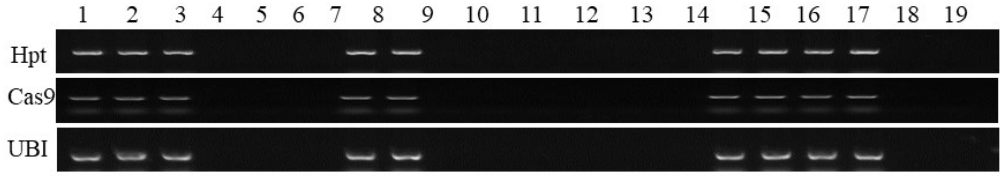

Figure 3. Molecular confirmation of T3 mutants. Note: Hpt (Hygromycin gene), Cas9 (Cas9 gene) and UBI (promotor sequence) are all located on the pREGB32 vector.1–19 were T3 generation plants, respectively. Primers in Supplementary Table S1.

3.3. Predicted Amino Acid Sequence of Rc Gene Mutant

Using *Rc* from weedy rice and the reported five *Rc* alleles as controls, the red pericarp of the mutants (*Rc* 1-162-1, *Rc* 2-162-16 and *Rc* 2-162-17) in T1–T3 generations was found to change from red to white due to the mutation of *Rc* gene that destructed the bHLH region, while the pericarp color of mutant (*Rc* 1-162-18) in T1–T3 generations was still red because the *Rc* mutation did not affect the bHLH region (Table 2).

Table 2. Predicted amino acid sequence analysis of *Rc* mutants of weedy rice using 5 kinds of *Rc/rc* alleles as control.

Samples	bHLH Region	Pericarp Color
WRL-162	ESRGGKGASGTRKVGAIQGDFSANHVLKERRRREKLNEKFI ILRSLVPFMTKMDKASILGDTIEYVKQLRNRIQELE	Red
Rc 1-162-T1-18	ESRGGKSGTRKVGAIQGDFSANHVLKERRRREKLNEKF IILRSLVPFMTKMDKASILGDTIEYVKQLRNRIQELE	Red
Rc 1-162-T1-1	ESRGGKGEKSVPSKVISVPTMC*KRGEEERSSMRSS* FCDLWYLS*QRWTRRRY*ATRSST*SS*GTAYKSSSRRRRRHE	White
Rc 2-162-T1-16	ESRGGKGAKWNAKSRCHPRFQCQPCAEREEKKREAQ EVHNSAIFGTFHDKDGQGVDTRRHDRVREAAKEPHTR	White

Table 2. *Cont.*

Samples	bHLH Region	Pericarp Color
Rc 2-162-T1-17	ESRGGKGASGTRKVGAIQGDFSANHVLKREEKKREAQ.EVH NSAIFGTFHDKDGQGVDTRRHDRVREAAKEPHT	White
Rc-r (Perla)	EQKHLNQGAGKAQVDAIQGDFSANHVLKERRRREK LNEKFIILRSLVPFMTKMDKASILGDTIEYVKQLRNRIQELE	Red
Rc (H75)	ESRGGKGASGTRKVGAIQGDFSANHVLKERRRRE KLNEKFIILRSLVPFMTKMDKASILGDTIEYVKQLRNRIQELE	Red
Rc (*Oryza rufipogon*)	ESRGGKGASGTRKVGAIQGDFSANHVLKERRRREKLN EKFIILRSLVPFMTKMDKASILGDTIEYVKQLRNRIQELE	Red
Rc g (WELL mutant)	ESRAGKAQVDAIQGDFSANHVLKERRRREK LNEKFIILRSLVPFMTKMDKASILGDTIEYVKQLRNRIQELE	Red
Rc-s (Surjamkuhi)	ESRGGKGASGTRKVGAIQGDFSANHVLKERRRRE KLNEKFIILRSLVPFMTKMDKASILGDTIEYVKQLRNRIQELE	Red
rc (Nipponbare)	ESRGGKGASGCHPRFQCQPCAEREEKKREA QEVHNSAIFGTFHDKDGQGVDTRRHDRVREAAKEPHTRARV	White
rc (Jefferson)	ESRGGKGASGCHPRFQCQPCAEREEKKREAQE VHNSAIFGTFHDKDGQGVDTRRHDRVREAAKEPHTRARV	White
rc (WELL)	ESRGGKGASGCHPRFQCQPCAEREEKKREAQ EVHNSAIFGTFHDKDGQGVDTRRHDRVREAAKEPHTRARV	White

Note: The green and yellow regions are the target amino acids of pRGEB32-Rc 1 (RGGKGA) and pRGEB32-*Rc* 2 (ANHVLKE), respectively. The blue region is 14 bp region (TRKVG), which is deleted in *rc* compared to *Rc*. The red region is the amino acid sequence of the bHLH domain. *Rc-r* (Perla) from Lee et al., 2009 [23]; *Rc* (H75) from Sweeney et al., 2006 [7]; *Rc* (*Oryza rufipogon*) from Sweeney et al., 2006 [7]; *Rc g* (WELL mutant) and *rc* (WELL) from Brooks et al., 2008 [22]; *rc* (Jefferson) from Sweeney et al., 2006 [7]; *Rc-s* (Surjamkuhi) from Sweeney et al., 2007 [8].

3.4. Off-Target Detection of Rc Mutant

Two potential off-target sites were screened using the CRISPR RGEN Tools (accessed on 15 June 2019; http://www.rgenome.net/) (Supplementary Table S2). PAM type is SpCas9 from Streptococcus pyogenes. The target genome was *Oryza sativa* (OSv4). Then, input Query Sequences (Rc1 and Rc2). Mismatch Number was set to less than 4. The two potential off-target sites were found. Then, the whole genome sequence of rice was downloaded from NCBI, and 1000 bp covering the potential off-target sites copied. PCR primers were designed to sequence using Primer 5.0 and identify all 9 *Rc* mutants, and none of the off-targets were found (Supplementary Table S3; Supplementary Figure S4).

3.5. Drought Tolerance in Seed Germination and Phenotype Traits of Wild Type and T3 Mutant of Weedy Rice

Using wild type and rice variety (Nipponbare) as control, the drought tolerance in seed germination and phenotype traits of T3 weedy rice mutants was measured. For seed germination with distilled water (Figure 4a), the germination number of the white pericarp mutants was significantly higher than that achieved for the wild type and rice variety (Nipponbare) on the second day. For seed germination with 20%PEG (Figure 4b), the germination number of the weedy rices was significantly higher than that achieved for the rice variety (Nipponbare) on the 5th day. For seeds pretreated in a 45 °C oven for 48 h and then germinated in distilled water (Figure 4c), the germination number of weedy rices was not significantly higher than that achieved for the rice variety (Nipponbare) on the 5th day. For seeds pretreated in a 45 °C oven for 48 h and then germinated in 20% PEG (Figure 4d), the germination number of the white pericarp mutants was significantly higher than that achieved for the wild type and rice variety (Nipponbare) on the 5th day.

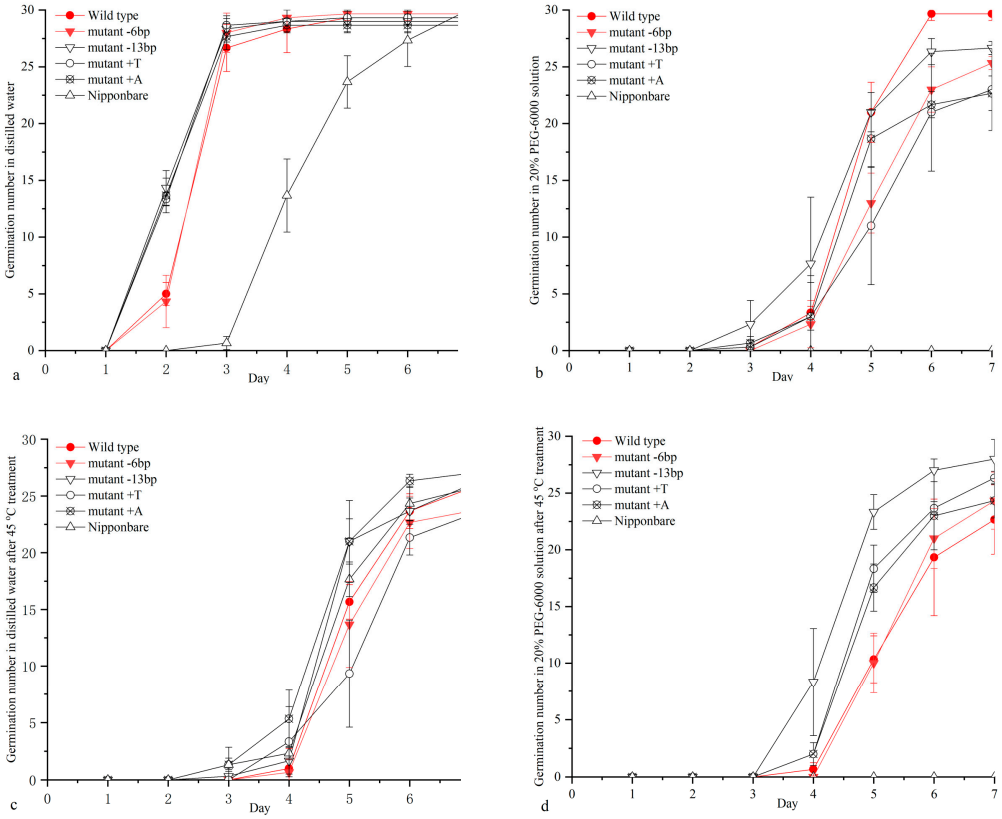

Figure 4. Drought tolerance in seed germination of wild type and T3 mutant of weedy rice. Note: Four treatments including with distilled water germination (**a**), 20%PEG-6000 drought-tolerant germination (**b**), distilled water germination after weak dormancy breaking (**c**), and 20%PEG-6000 drought-tolerant germination after weak dormancy breaking (**d**), respectively. Three-time replicates were carried out for seed germination, and for each replication, 30 seeds were used. Wild type is Wrl-162. Mutants are Rc1-162-18, Rc1-162-1, Rc1-162-16, and Rc1-162-17. Wild type weedy rice and rice cultivar (Nipponbare) as control.

Comparing the wild type with some mutant types with white pericarp in T3 generation weedy rice without CRISPR-Cas9 vector, there was no significant difference in panicle number, seed setting rate per plant, grain length and width (Figure 5a). However, some mutant types with white pericarp in T3 generation weedy rice without CRISPR-Cas9 vector registered significantly decreased plant height, decreased spikelet number per main panicle, decreased plump seed number per main panicle, and increased 1000-grain weight (Figure 5b).

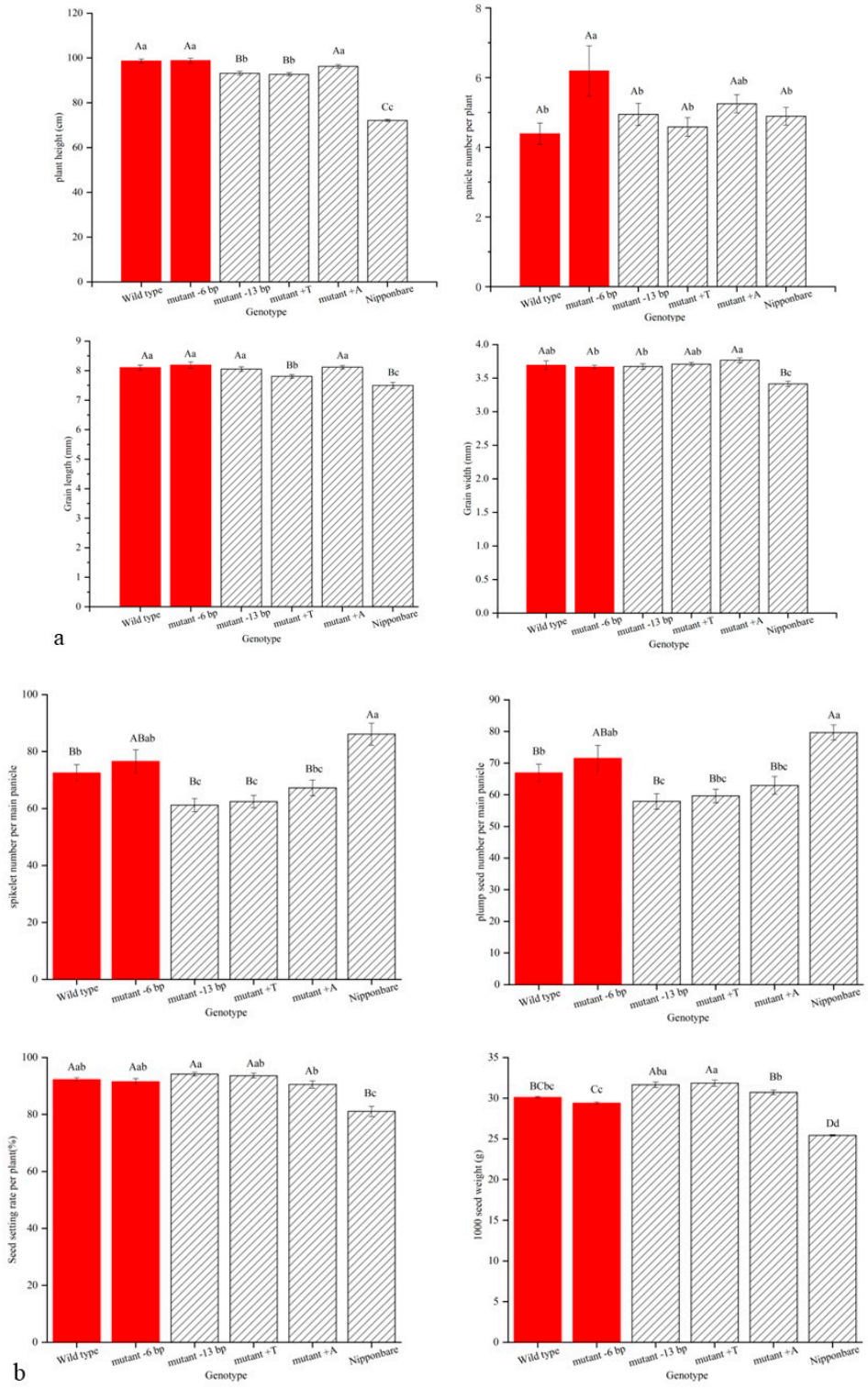

Figure 5. Phenotype traits of wild type and T3 mutant of weedy rice. Note: eight traits were measured including plant height, panicle number, grain length and width (**a**), seed setting rate per plant, spikelet number per main panicle, plump seed number per main panicle, and 1000-grain weight (**b**). Three-time replicates were carried out and for each replication, 12 plants were used. Wild type is Wrl-162. Mutants are Rc1-162-18, Rc1-162-1, Rc1-162-16, and Rc1-162-17. Wild type weedy rice and rice cultivar (Nipponbare) as control The vertical bars indicate standard error of the means.. Significance levels are indicated by letters (a, b, c) and letters (A, B, C) for $p < 0.05$ and $p < 0.01$, respectively.

Therefore, *Rc* gene mutation can not only change the pericarp color of weedy rice, but also affect the seed germination, drought resistance, and even the phenotypic characters of vegetative and reproductive periods.

4. Discussion

4.1. The bHLH Region of the Rc Gene Plays a Critical Role in Determining the Pericarp Color

The *Rc* gene of Japonica type possesses multiple SNPs which are different from that of indica and wild rice [39]. In this experiment, CRISPR/Cas9 technology was used to mutate the *Rc* gene in red pericarp of japonica-type weedy rice. Three weedy rice mutants (*Rc* 1-162-1, *Rc* 2-162-16, *Rc* 2-162-17) were obtained with pericarp color changing from red to white. The amino acid sequence of the bHLH region of *Rc* gene was analyzed, and it was found that the pericarp color transition of the weedy rice was dependent upon the presence of bHLH protein domain. The weedy rice mutants with changed pericarp color from red to white lost the bHLH region (Table 2). Therefore, the transformation of the bHLH protein domain can not only change the pericarp trait from white to red [28], but also from red to white, thus removing harmful traits of weedy rice.

4.2. Domestication of Weedy Rice into Direct-Seeding Rice Might Be Achieved by Precisely Editing a Few Unfavorable Genes

The red pericarp color of weedy rice is a significant character that harms rice quality. By precisely editing the *Rc* gene, the pericarp color of the weedy rice mutant was changed from red to white, and the drought resistance of seed germination was improved (Figure 4a). This might mean that gene editing broke the seed dormancy controlled by the *Rc* gene, thereby improving its germination ability, and thus improving the drought resistance of seed germination [39]. However, the drought resistance of the mutants was still significantly improved after the seed dormancy was eliminated with high temperature (Figure 4d) [31]. This suggests that there are pleiotropic effects in the red–white transformation of the pericarp color [40].

Zsögön (2017) [41] first put forward the concept of "de novo domestication", that is, manipulating wild species at the gene level to domesticate them de novo. Furthermore, de novo domestication of wild tomato was studied using genome editing [42,43]. Compared with the wild tomato parent, mutants can increase fruit size, fruit number, flower and ascorbic acid synthesis. Thus, de novo domestication of weedy rice should also be possible by editing genes to change the non-dispersion of seeds, gigantism, and through modifications on the plant's architecture [10,41].

Red pericarp, one of the most prominent traits of weedy rice, seriously affects the quality and commercial value of cultivated rice. As a first step in the domestication of weedy rice, we attempted to remove harmful pericarp trait. In addition, the domestication of weedy rice also helps to understand the effects of traits on weed persistence in farmland so as to better utilize and manage weedy rice as a germplasm resource.

5. Conclusions

The pericarp color of weedy rice could be transformed into white using CRISPR/Cas9 technology. The bHLH region of the *Rc* gene determines the red pericarp color. By precisely editing the *Rc* gene, not only was the important harmful trait of the red pericarp of weedy rice removed, but the drought resistance of seed germination was also improved. Thus, domestication of weedy rice toward direct-seeded rice could be achieved by precisely editing some disadvantaged genes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13041130/s1, Table S1: Primers in the experiment; Table S2: Potential off-target site detection; Table S3: Potential off-target sequencing primers; Figure S1: Construction of CRISPR/Cas9-Rc mutation vector; Figure S2: Sequence alignment of pRGEB32-Rc mutation vector and the original plasmid pRGEB32; Figure S3: Genetic transformation of Agrobacterium tumefaciens using seed mature embryo; Figure S4: Potential off-target sequence alignment.

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