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Breeding of *Indica* glutinous cytoplasmic male sterile line WX209A *via* CRISPR/Cas9 mediated genomic editing

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Abstract: Glutinous cytoplasmic male sterile (CMS) line is necessary to select hybrid glutinous rice combination with high yield and quality. To develop glutinous CMS with low amylose content, in this study, we firstly knocked out the granule-bound starch synthase *OsWaxy* in 209B using CRISPR/Cas9 mediated genome editing technology and successfully obtained a glutinous maintainer line WX209B. Comparing with maintainer line 209B, WX209B showed decreased amylose contents and similar agronomic characters. And then, through one generation of hybridization and two generations of backcrossing with WX209B as the male parent and 209A as the female parent, the glutinous CMS line WX209A was successfully achieved. Our study provides a strategy to efficiently breed for the glutinous cytoplasmic male sterile line by combining CRISPR/Cas9-mediated gene editing technology with conventional backcross breeding method in a short period, which prepares the ground for further breeding of hybrid glutinous rice variety.

Keywords: glutinous rice; CRISPR; *OsWaxy*; rice breeding

Glutinous rice (*Oryza sativa* var. *glutinosae*) is a glutinosity type of rice variety with high edible and economic value. Because the low yield and poor quality of conventional glutinous rice varieties, it is very important to develop hybrid glutinous rice varieties. To breed hybrid glutinous rice variety with high yield and quality, both parents should be glutinous. Therefore, the breeding of glutinous cytoplasmic male sterile line (CMS) is necessary to select new hybrid glutinous rice combination. However, it is long-running and difficult to breed glutinous CMS lines by traditional advanced-backcross method, so it is necessary to introduce new technology in the traditional breeding process. Compared with non-glutinous rice, glutinous rice lacks the amylose. *OsWaxy* encoding a granule-bound starch synthase is responsible for the change of amylose content (Wang *et al*. 1990), the loss of *OsWaxy* function causes the decrease in amylose content. Therefore, the knockout of *OsWaxy* is crucial to decrease amylose content and breed glutinous CMS lines.

The recently developed CRISPR/Cas9 system has been demonstrated to be effective tool for targeted genome editing. This technology employs a Cas9 endonuclease and a guide RNA complex to generate mutations in specific target genes. So far, this technology has been successfully applied in targeted genome editing in yeast, mouse, zebra fish, human

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cell lines, Arabidopsis, rice, tobacco, wheat, sorghum and maize (Cong *et al*. 2013; DiCarlo *et al*. 2013; Feng *et al*. 2013; Hwang *et al*. 2013; Jiang *et al*. 2013; Wang *et al*. 2013, 2014a, b; Liang *et al*. 2014; Ma *et al*. 2015). In rice, the CRISPR/Cas9 system was first successfully used to create targeted mutations in both *OsPDS-P1* and *OsBADH2* (Shan *et al*. 2013). Nowadays, CRISPR/Cas9-mediated gene editing in rice has also been successfully achieved in *CAO1*, *LAZY1*, *PDS*, *PMS3*, *OsEPSPS*, *OsDERF1*, *OsMSH1*, *OsMYB1*, *OsMYB5*, *OsROC5*, *OsSPP*, *OsYSA*, *AOX1*, *OsWaxy*, *OsGSTU*, *OsMRP15*, *OsAnP*, *Gn1a*, *DEP1*, *GS3*, and *IPA1* (Miao *et al*. 2013; Shan *et al*. 2014; Zhang *et al*. 2014; Xu *et al*. 2015; Ma *et al*. 2015; Li *et al*. 2016), respectively. However, the evaluation of the breeding value of related mutants is rare.

In this study, through CRISPR/Cas9 mediated genome editing, we knocked out the *OsWaxy* in 209B, the maintainer line of cytoplasmic male sterile line 209A, and obtained a mutant defined as WX209B, which showed lower starch amylose content and similar agronomic characters to 209B. Through one generation of hybridization and two generations of backcrossing with WX209B as the male parent and 209A as female parent, the glutinous CMS line WX209A was successfully achieved. This study enables the decrease in starch amylose content of WX209A, thus effectively shortening the breeding time of hybrid glutinous rice varieties.

MATERIAL AND METHODS

Plant materials, vectors and strain. The *indica* cytoplasmic male sterile line 209A and its maintainer line 209B were provided by Guangxi WuTai Seed Co, Ltd. All materials were grown in paddy fields under normal growth conditions. The sgRNA-Cas9 plant expression vectors were kindly provided by Professor Yaoguang Liu (South China Agricultural University). PMD19-T was purchased from Takara Biotechnology Co, Ltd. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* EHA105 were maintained by our laboratory.

Vector construction. The sgRNA-Cas9 plant expression vectors were constructed as previously described (Ma *et al*. 2015). The oligos used in constructing the sgRNA vectors for *OsWaxy* were found in Ma *et al*. (2015).

Rice transformation. The CRISPR/Cas9 constructs were introduced into *A. tumefaciens* EHA105 by electroporation, rice transformation was performed by the *A. tumefaciens*-mediated transformation method (Hiei *et al.* 1994). Transgenic rice plants were selected on hygromycin medium. T_0 transgenic rice plants were used for the detection of mutations.

Target site mutation detection of T_0 plants. By the cetyltrimethyl ammonium bromide (CTAB) method (Rowland & Nguyen 1993), genomic DNA was extracted from T_0 transgenic rice plants. The genomic region surrounding the CRISPR target sites was amplified by PCR reaction using the specific primers 5'-TCCGCCACGGGTTCCAG-3' (*OsWaxy*-test-F) and 5'-CGTTGTGGCTGAGGTAGGAG-3' (*OsWaxy*-test-R). The PCR products were directly sequenced. The mutations were detected using Degenerate Sequence Decoding method (Ma *et al*. 2015).

Detection of T-DNA-free T_1 mutant plants. T_1 plants derived from the self-bred progenies of bi-allelic and homozygous T_0 mutant plants were used to screen the T-DNA-free T_1 mutation. By the CTAB method, genomic DNA was extracted from T_1 transgenic rice plants. PCR was performed to identify T-DNA-free T_1 mutant plants using Cas9 gene specific primer 5'-CTGACGCTAACCTCGACAAG-3' (Cas9-F) and 5'-CCGATCTAGTAACATAGATGACACC-3' (Cas9-R). The PCR products were analyzed by gel electrophoresis on 1% agarose gels. The plants which could not amplify target bands were T-DNA-free T_1 mutant rice plants.

Measurement of amylose and amylopectin contents. Amylose contents of rice seeds were measured as previously described (Perez & Juliano 1978), with three replicates for each sample.

Investigation of main agronomic characters. Five T_0 and T_1 homozygous mutant plants were selected to investigate the main characters, respectively. The main characters included plant height, flag leaf length, flat leaf width, panicle length, set grain rate, and sterile grain rate. 209B was used to as control.

Development of glutinous sterile line WX209A. The CMS line, 209A, as female parent, was crossed and backcrossed with the maintainer 209B - derived T_1 homozygous mutant glutinous plants (WX209B). The homozygous glutinous CMS plants (WX209A) were selected by marker-assisted selection with target site specific primers OsWaxy-test-F and OsWaxy-test-R in the BC_1 generation. The selected WX209A were backcrossed with the WX209B again to multiply the seeds of the CMS line.

RESULTS

209B-*OsWaxy* target site primer. It was reported that the CRISPR/Cas9 technique was successfully

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used to create targeted mutations in *OsWaxy* gene of Taichung 65 (T65), which decreased the amylose content from 14.6% in T65 to 2.6% in the mutants (Ma *et al.* 2015). Comparing the *OsWaxy* target sites sequence between 209B and T65, we found that they are the same (Figure 1A). Therefore, the target site primers of *OsWaxy* in T65 were introduced to our study. The target site primers of *OsWaxy* were as followed: 5'-GCCGTGTGTGCTTACAGCCATGGC-3'(*OsWaxy*-U6-F and 5'-AAACGCCATGGCTGTAA-GCACACA-3'(*OsWaxy*-U6-R).

Identification of pYLCRISPR/Cas9-OsWaxy-sgRNA expression vector. To identify whether pYLCRISPR/Cas9-OsWaxy-sgRNA expression vector was successfully constructed, PCR was performed by using the consensus primer 5'-CTCCGTTTTAC-CTGTGGAATCG3' (U-F) and corresponding target site adaptor primer 5'-AAACGCCATGGCTGTAA-GCACACA3' (OsWaxy-U6-R). The result showed that the size of PCR product was 629 bp, which was consistent with that of sgRNA-osU6a fragment (Figure 1B). This suggests that pYLCRISPR/Cas9-OsWaxy-sgRNA expression vector is successfully constructed and is suitable to be transformed into 209B by the *A. tumefaciens*-mediated transformation method.

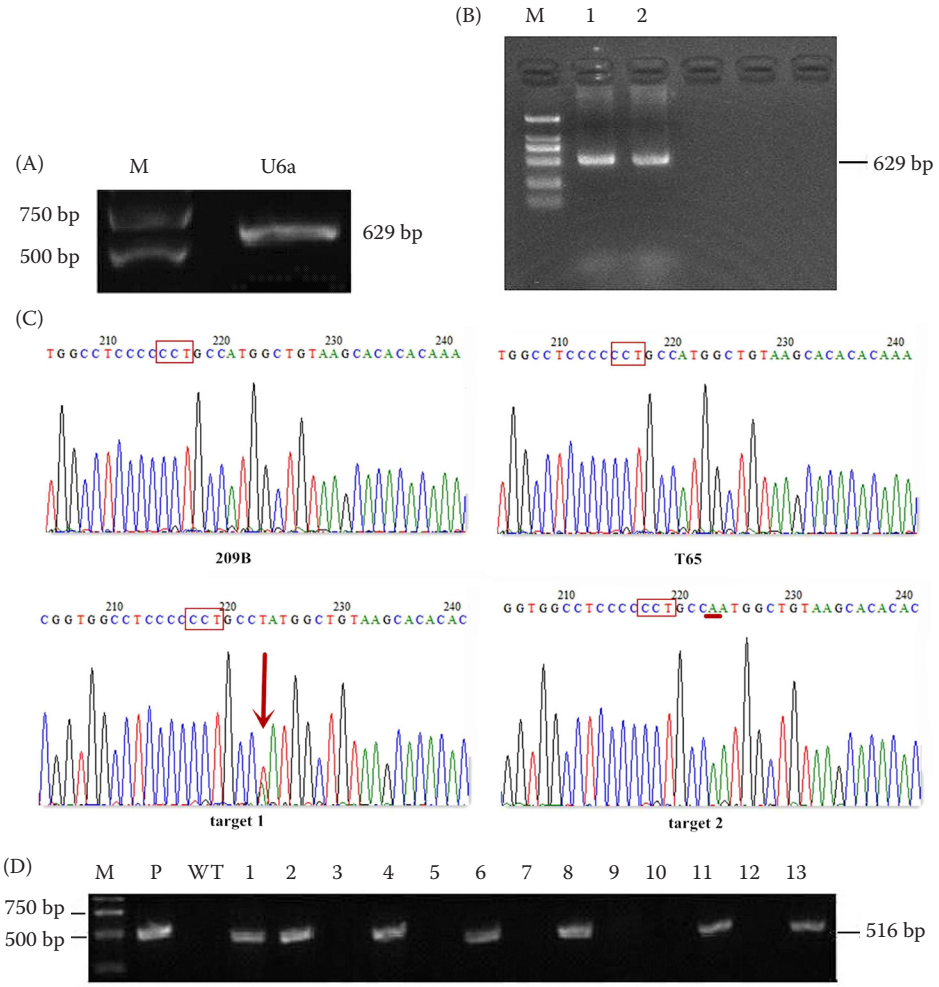

Figure 1. Detection of mutations in *OsWaxy* gene by CRISPR/Cas9-mediated genome editing: (A) construction of OsWaxy-sgRNA expression vector; (B) identification of pYLCRISPR/Cas9-OsWaxy-sgRNA expression vector; M – D2000 marker; 1~2 are pYLCRISPR/Cas9-OsWaxy-sgRNA expression vector; (C) mutation types at *OsWaxy* loci in T_0 generation; target 1 – biallelic mutant; target 2 – homozygous mutant; target; red box indicates protospacer adjacent motif (PAM); the red arrow is biallelic mutation site; (D) detection of T-DNA-free T_1 homozygous mutant plants; M – marker; P – positive control; WT – negative control; lane No. 1~6 are mutation plants of the allelic mutation line 2-1; lane No. 7~13 are mutation plants of the homozygous mutation line 2-6; the targeted 516 bp band is the band pattern with pYLCRISPR/Cas9 vector of T_1 homozygous mutant plants, no band is the band pattern of T-DNA-free T_1 homozygous mutant plants

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Table 1. Mutation rate of target locus in transgenic T_0 rice plants

T_0 transgenic plants	Mutation type					Total
	biallelic	homozygous	heterozygous	chimeric	wild	
No. of plant	8	7	2	1	8	26
Mutation rate (%)	31.8	26.9	7.7	3.8	31.8	100

Analysis of mutation rate of target locus in transgenic T_0 plants. The mutation of target sites in transgenic T_0 plants was determined by PCR reaction and direct DNA sequencing. The mutant genotype and DNA base change in target site are shown in Table 1 and Figure 1C, respectively. In total, 18 T_0 mutant plants were obtained from 26 T_0 plants. This suggests that the mutation frequency of target site is 69.2%. Of these 18 T_0 mutant plants, the frequency for homozygous, biallelic, heterozygous, chimeric mutant plants were 26.9%, 31.8%, 7.7% and 3.8%, respectively. These suggest that homozygous mutation frequency of target site is sufficiently high for further screening.

Detection of T-DNA-free plants in T_1 generation. To obtain the T-DNA-free T_1 mutant plants, T_1 plants derived by self-fertilisation of biallelic and homozygous T_0 mutant plants were determined by PCR reaction and direct DNA sequencing. The result shows that, of homozygous and biallelic T_1 mutant plants, the frequencies of T-DNA-free plants were 21.1% and 22.3%, respectively (Table 2 and Figure 1D).

Analysis of amylose content in T_0 mutant plants. Amylose content is one of the key factors determining the grain quality in rice, the amylose content of glutinous rice varieties is generally very low. To determine the effect of *OsWaxy* mutation on the grain quality, 22 T_0 mutant plants were selected to analyze the amylose content. The result shows that, of 22 tested T_0 mutant plants, the amylose contents of 6 plants, such as 2-4 (2.6%), 2-6 (3.6%), 2-14 (3.8%), 2-17 (2.4%), 2-19 (1.2%) and 2-25 (2.0%), were significantly lower than the control 209B (12.8%), but similar to those of 3 local traditional glutinous rice varieties, such as big glutinous rice (1.8%), shangsi glutinous rice (1.0%), and jingxi glutinous rice (3.6%). These 6 plants with low amylose contents accounted for 27.3% of total tested T_0 mutant plants. Sixteen plants including 2-1, 2-3, 2-5, 2-7, 2-8, 2-9, 2-10, 2-11, 2-12, 2-13, 2-15, 2-16, 2-18, 2-20, 2-21 and 2-24, displayed similar amylose contents to the control 209B (12.8%), These 16 plants with similar amylose contents to the control 209B accounted for 72.6% of total T_0 tested plants. In addition, we found that

Table 2. Detection for T-DNA-free mutation rice plants in T_1 generation

	Plant type		
	wild	biallelic	homozygous
No. of tested plants	64	103	95
No. of T-DNA-free plants	64	23	20
Frequency of T-DNA-free plants (%)	100.0	22.3	21.1

Table 3. Amylose content of rice T_0 and T_1 mutant plants (average of three duplicates)

T_0 plant	Amylose content (%)	T_1 homozygous mutation plant	Amylose content (%)
2-1	12.6	2-4-1	2.8**
2-3	11.0	2-4-2	2.2**
2-4	2.6**	2-4-3	2.4**
2-5	12.2	2-4-4	2.8**
2-6	3.6**	2-6-1	3.2**
2-7	12.5	2-6-2	3.4**
2-8	13.3	2-6-3	3.0**
2-9	12.2	2-6-4	2.8**
2-10	12.7	2-17-1	2.2**
2-11	12.0	2-17-2	2.0**
2-12	11.8	2-17-3	2.6**
2-13	12.3	2-17-4	2.2**
2-14	3.8**	2-19-1	1.8**
2-15	11.8	2-19-2	1.0**
2-16	12.2	2-19-3	1.6**
2-17	2.4**	2-19-4	1.5**
2-18	10.6	2-25-1	1.8**
2-19	1.2**	2-25-2	1.8**
2-20	12.0	2-25-3	2.2**
2-21	13.7	2-25-4	2.4**
2-24	13.2	big glutinous rice	1.8**
2-25	2.0**	shangsi glutinous rice	1.0**
209B (control)	12.8	jingxi glutinous rice	3.6**
		209B (control)	12.8

**significant differences at $P < 0.01$

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Table 4. Main agronomic characters in rice T_0 homozygous mutants

T_0 homozygous mutant plant	Plant height (cm)	No. of panicles	Flag leaf length	Flag leaf width	Length of panicle
			(cm)		
209B	88.2	6	52.2	1.5	24.6
2-4	88.0	6	50.4	1.5	24.0
2-6	88.6	5	48.8	1.5	23.7
2-17	81.8	5	54.0	1.5	26.3
2-19	88.2	5	53.6	1.4	25.9
2-25	89.2	6	50.4	1.5	24.6

all these 6 plants with low amylose contents are T_0 homozygous mutant plants. This suggests that, of 7 T_0 homozygous mutant plants, only 1 plants had not reached the level of the glutinous rice (below 4 %) in amylose content. At the same time, the fact that other type of T_0 mutant plants are not subjected to change of amylose content suggests that those mutations of *OsWaxy* in other type of T_0 mutant plants may not cause the functional loss of OsWaxy protein.

Analysis of amylose content in T_1 homozygous mutation plants. Five T-DNA-free T_0 homozygous mutant plants with low amylose content were selected to self-fertilized to produce T_1 lines and 4 plants of each T_1 line were selected to determine the amylose content. This result showed that the amylose contents of all 20 plant samples are below 3.4%, which were consistent with those of 3 glutinous rice varieties with amylose contents ranging from 1% to 3.6%. This indicates that the homozygous glutinous mutation character of T_0 mutant plants can be steadily inherited to T_1 mutant plants (Table 3). And what is more, all these 20 T_1 homozygous mutants are T-DNA-free plants, which provides useful middle materials to breed hybrid glutinous rice varieties.

Investigation of main agronomic characters of T_0 and T_1 homozygous mutant plants. To elucidate the effect of *OsWaxy* mutation on main agronomic characters, we investigated the main agronomic characters (including plant height, panicle number, flag leaf length, flag leaf width, and panicle length) of 5 T_0 homozygous mutant lines grown in paddy fields under normal growth conditions. This result showed that all 5 T_0 homozygous mutant lines displayed similar phenotypes to the control 209B, except for the insignificant difference of individual character which may be caused by different water and fertility

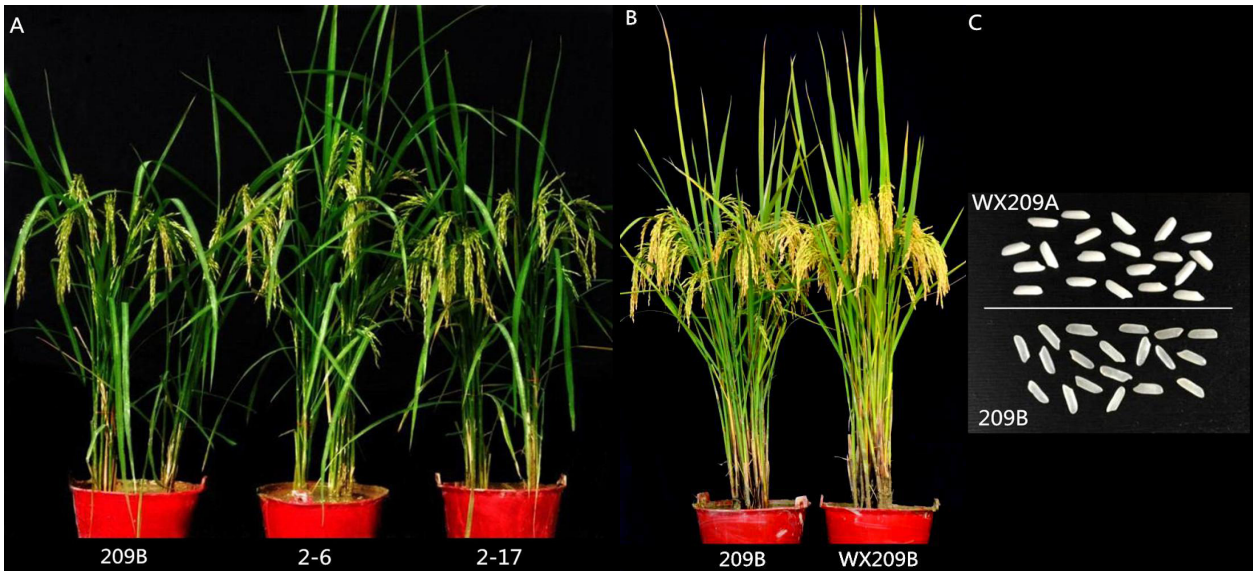

Figure 2. Comparison of T_0 homozygous rice mutants with parent 209B: plants 2-6 and 2-17 are homozygous mutant type, 209B is receptor parent (A); comparison of plant type of T_1 T-DNA-free homozygous mutant line WX209B (right) with 209B (left) (B); comparison of polished rice between glutinous WX209A (top) and non-glutinous 209B (below) (C)

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Table 5. Main agronomic characters in T-DNA-free rice T_1 homozygous mutants

T_1 homozygous mutant plant	Plant height (cm)	No. of panicle	Flag leaf length	Flag leaf width	Length of panicle	Grain No. per spike	Grain set rate (%)
			(cm)				
209B	84	9.4	41.8	1.5	24.2	188	86.5
2-4	83	8.8	42.3	1.5	24.0	182	84.6
2-6	85	9.6	38.5	1.5	24.2	196	85.0
2-17	83	9.6	45.2	1.5	23.8	186	85.2
2-19	80	9.0	45.5	1.5	24.8	180	86.0
2-25	86	9.0	43.3	1.5	24.0	190	84.2

condition (Figure 2A, Table 4). Data from 5 T-DNA-free T_1 homozygous mutant lines show that there was no significant difference in 7 main agronomic characters (including plant height, panicle number, flag leaf length, flag leaf width, panicle length, grain number of per spike and seed setting rate) in these T_1 plants compared to the control 209B (Figure 2B, Table 5). This indicates that the homozygous mutation in *OsWaxy* had no significant effect on main agronomic characters and T_0 phenotypes can be steadily inherited to T_1 generation. In addition, we found that both T_1 plants and the control 209B showed stronger stem, stronger tillering ability, larger panicle and more grain numbers than T_0 plants (Table 5). This phenotypic difference between T_0 and T_1 generation may be caused by different water and fertility condition. Among 5 T-DNA-free T_1 homozygous mutant lines, 2-6 line, denominated as WX209B, was used to further breed glutinous CMS line.

Development of glutinous sterile line WX209A. To speed up the breeding process of glutinous sterile lines, WX209B (as the male parent) was used to hybridize with cytoplasmic male sterile lines 209A (as female parent) to produce F_1 hybrids, and then the F_1 hybrids were backcrossed with WX209B. By molecular marker-assisted selection (MAS), the homozygous BC_1F_1 CMS plants were selected to continuously backcross with WX209B to obtain the homozygous BC_2F_1 CMS line, which is glutinous and no genetic segregation (Figure 2C). Therefore, this glutinous CMS line selected from BC_2F_1 is denominated as WX209A and used for the further breeding of hybrid glutinous rice variety.

DISCUSSION

Glutinous rice is a kind of food crop with high nutritive value and the important material in food processing industry. Comparing with non-glutinous rice, it is more valuable to improve the quality of glutinous rice. Amylose content is one of the key factors in determining the quality of glutinous rice and the amylose content of conventional glutinous rice varieties is generally very low. Although some glutinous rice varieties with low amylose content were obtained by CRISPR/Cas9-mediated gene editing technology, they are difficult to be applied to practical production due to the low yield or poor quality. So, it is very important to breed hybrid glutinous rice variety with high yield and quality. Comparing with conventional backcross breeding method, CRISPR/Cas9-mediated gene editing technology not only can shorten breeding periods, but also can produce clean plants without transgene in one or two generations. Therefore, the combination of CRISPR/Cas9-mediated gene editing technology and conventional backcross breeding method could greatly increase breeding efficiency of hybrid glutinous rice variety, which present a better alternative strategy to breed for the glutinous rice varieties with low amylose content, high yield, and quality.

Regarding the directed editing of rice *OsWaxy*, Ma *et al.* (2015) used the CRISPR/Cas9 gene editing system to design three target sites between exons 1, 4, 5, 6, and 7, respectively, using the corresponding U6a, U3, and U6b promoter, Agrobacterium-mediated transformation of T65, yielded 3 independent mutant strains (OS-1~OS-3). Rice with amylopectin content decreased from 14.6% to 2.6% showed a glutinous nature. The results showed that the expression level of *OsWaxy* in the mutant strain was significantly reduced, and the amylose content in rice was significantly reduced. Such as the same research results.

Prior to the maturation of gene editing technology, improved use of antisense RNA to reduce *OsWaxy* or RNAi technology silences *OsWaxy*. Terada *et*

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al. (2000) transformed the antisense *OsWaxy* into *japonica* rice and *indica* rice and obtained transgenic mutant lines with significantly reduced amylose content.

In this research, first of all, a glutinous maintainer line WX209B was created by CRISPR/Cas9-mediated gene editing technology. And then, through one generation of hybridization and two generations of backcrossing with WX209B as the male parent and 209A as female parent, the glutinous cytoplasmic male sterile lines (CMS) WX209A was successfully achieved. The analysis of mutation rate of target locus in T_0 plants showed that the mutation frequency of target site is 69.2%, suggesting that the off-target mutation frequency of *OsWaxy* locus is very low. It is noteworthy that the frequency of *OsWaxy* homozygous deletion mutation accounted for 26.9%, indicating that CRISPR/Cas9-mediated gene editing technology was very useful for obtaining homozygous deletion mutants in T_0 plants. The analysis of T-DNA-free plants of T_1 generation showed that, of homozygous T_1 mutant plants, the frequencies of T-DNA-free plants were 21.1 %, this provides enough materials for the selection of clean plants without transgene. At the same time, the analysis of amylose content and main agronomic characters of T_0 and T_1 homozygous mutation plants showed that the amylose contents and main agronomic characters of T_0 and T_1 homozygous mutation plants were similar, this suggests that mutation of *OsWaxy* were highly stable and can be inherited from T_0 to T_1 generation.

In brief, our study provides a strategy to efficiently breed for the glutinous maintainer line and sterile line in short period, which prepare the ground for further breeding of hybrid glutinous rice variety.

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