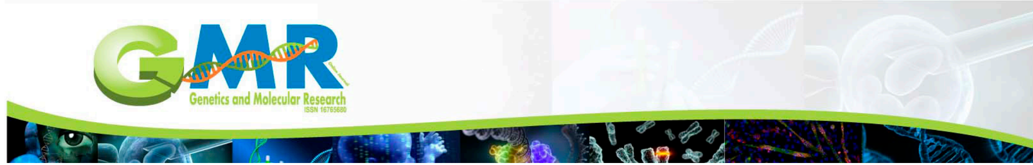

Analysis of specific sequences in mutant rice generated by introduction of exogenous corn DNA

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Genet. Mol. Res. 14 (4): 18980-18989 (2015)
Received September 5, 2015
Accepted November 11, 2015
Published December 29, 2015
DOI http://dx.doi.org/10.4238/2015.December.29.5

ABSTRACT. Rice variation induced by the introduction of exogenous DNA has become an important method of improving rice varieties and creating new germplasms. In this study, we transferred maize genomic DNA fragments to the receptor of Nipponbare rice using a modified "pollen-tube pathway" method. Material from mutant rice B_1 and B_2 were acquired and 14 specific bands were obtained from the material using amplified fragment length polymorphism analysis. From the 14 specific sequences obtained, there were 3791 bp, including 144 base mutations with a base mutation rate of 3.80%. Specific bands resulted from base mutation of selective bases or restriction endonuclease recognition sequences, or insertion or deletion of DNA fragments. The frequency of single-base mutations was significantly higher than that of double-base mutations, three-sequential base mutations, and multiple-sequential base mutations. The site frequency of base substitution (87.04%) was significantly higher than that of base insertion (3.70%) or deletion (9.26%). In all cases of base substitution, the frequency of transition (76.47%) was significantly higher than transversion (23.53%). The above results indicate that transferring foreign-species DNA into rice cells can induce base mutations in the receptor, with base

substitutions occurring at the highest frequency, and the dominant type of base substitutions being transition. Preliminary analysis reveals that the molecular mechanism of transferring exogenous DNA into rice causes mutations, which provides theoretical data on biological mutagenesis for further research.

Key words: Rice; DNA; Mutation; Base mutation

INTRODUCTION

Rice variation induced by the introduction of exogenous DNA has become an important method of improving rice varieties and creating new germplasms (Zhou et al., 2001; Tian et al., 2006; Ji et al., 2012). Zhao et al. (2001) introduced exogenous DNA from *Oryza minuta* into rice V20B using spike and stalk injection. After random amplified polymorphic DNA (RAPD) analysis, a highly similar DNA specificity-sequence was observed in the variant rice (V20B), demonstrating that the fragment of foreign DNA can be integrated into the genome of the receptor rice. At the same time, Zhao et al. (2001) also discovered some base mutations in the fragment of foreign DNA. Luo et al. (2012) hybridized *Zizania* with rice and obtained 3 stability variant lines through generational self-crosses. After amplified fragment length polymorphism (AFLP) analysis, gel electrophoresis, and DNA sequencing, they found that exogenous DNA might cause changes in the genome of the receptor (Luo et al., 2012). Sun et al. (2013) also introduced exogenous DNA from *O. minuta* into rice V20B using spike and stalk injection. Using whole genome sequencing, they found 27 genes corresponding to relevant variant traits and some mutations arising in these genes in the V20B rice (Sun et al., 2013). Wang et al. (2013) hybridized *Zizania* with rice and obtained a stability variant line (RZ35). Using whole genome sequencing, they discovered a high number of SNPs, insertions, and deletions in RZ35 (Wang et al., 2013). However, in all of the above studies, little was reported regarding the types of mutations occurring and the mutation sites. Moreover, there is currently no understanding of the reasons behind mutations induced by exogenous DNA at the molecular level.

In the present study, we transferred corn DNA to rice using an improved pollen-tube pathway method to gain novel genetic variant rice. AFLP markers were applied to analyze polymorphism in the two mutant rice variants. DNA sequencing was then used to analyze the cause of these specific bands, to explore the molecular mechanism of rice variation induced by exogenous DNA, and to provide a reference for further study of the mechanisms of biological mutations.

MATERIAL AND METHODS

Material

Exogenous corn DNA (*Zea mays* L. Zhengdan14; CK_1) was provided by the Henan Academy of Agricultural Sciences. The receptor rice (*Oryza sativa* L. Nipponbare; CK_2) was provided by the Xinxiang Academy of Agricultural Sciences. In 2010, corn DNA was transferred to the rice using an improved pollen-tube pathway method (Guan et al. 2014) to gain novel genetic variant rice. Two mutants (B_1, B_2) were then selected from the F1 generation and F3 seeds were obtained by generational self-crosses.

Extraction of genomic DNA

Genomic DNA was extracted from etiolated plants (F3) for AFLP analysis using a plant genomic DNA extraction kit (TaKaRa, Dalian, China) following the manufacturer protocol. The purity and concentration of the DNA were assessed using 8% agarose gel electrophoresis and a spectrophotometer. Samples were stored at -20°C until required.

AFLP analysis

Based on the optimized reaction and amplification conditions for AFLP analysis described by Vos et al. (1995) and Wei (2013), the adaptor and primer sequences used in the current study are shown in Table 1. We selected 12 pairs of optimized primers (P1M1, P1M2, P1M3, P2M4, P3M1, P3M5, P4M6, P4M8, P5M4, P7M1, P8M3, P8M4) from 64 pairs of AFLP primers to amplify the corn and rice samples (CK_1, CK_2, B_1, and B_2). All the amplified products were analyzed using polymorphic map analysis.

Table 1. Sequences of amplified fragment length polymorphism (AFLP) adaptors and primers used in the analysis of exogenous corn (*Zea mays*; CK_1), receptor rice (*Oryza sativa*; CK_2), and two mutant F3 rice lines (B_1, B_2).

Adapter and primer	Sequence (5' to 3')
*Pst*I adapter	CTCGTAGACTGCGTACATGCA
	TGTACGCAGTCTAC
*Mse*I adapter	GACGATGAGTCCTGAG
	TACTCAGGACTCAT
*Pst*I pre-amplification primer	GAC TGC GTA CAT GCA GA
*Mse*I pre-amplification primer	GAT GAG TCC TGA GTA AC
Selective amplification primers	Po: GAC TGC GTA CAT GCA G
	Mo: GAT GAG TCC TGA GTA A
*Pst*I selective amplification primer	P1: Po-AAC, P2: Po-AAG, P3: Po-ACA,
	P4: Po-ACC, P5: Po-ACG, P6: Po-ACT,
	P7: Po-AGC, P8: Po-AGG
*Mse*I selective amplification primer	M1: Mo-CAA, M2: Mo-CAC, M3: Mo-CAG,
	M4: Mo-CAT, M5: Mo-CTA, M6: Mo-CTC,
	M7: Mo-CTG, M8: Mo-CTT

Purification of specific bands

Specific bands were cut from the polyacrylamide gels for CK_1, B_1, and B_2, but not CK_2. The bands were placed in a 1.5-mL centrifuge tube and washed with 100 µL double-distilled water (ddH_2O). The ddH_2O was discarded and the gel was then steeped in 10 mL ddH_2O for 5 min. The ddH_2O was discarded again, the gel was crushed, and another 60 µL ddH_2O was added and the gel was steeped for 5 h. The centrifuge tube was then placed in boiling water for 15 min, followed by centrifugation at 12,000 *g* for 15 min. The supernatant was used as a template in PCR. PCR amplification was conducted using the pure DNA fragment and amplified products were electrophoresed on a 1.2% agarose gel. Specific bands were extracted from the agarose gels under ultraviolet light using a DNA gel extraction kit (TaKaRa), following the manufacturer protocol.

Cloning, sequencing, and alignment of specific bands

Amplified DNA fragments were recovered, inserted into pMD19-T plasmids, and the recombinant plasmids were transformed into competent *Escherichia coli* cells. Positive clones

were selected through blue and white screening and then added to cell media (Luria Broth with 100 mg/mL ampicillin) and cultured for 3 to 4 h. The cultured bacterial solution was sequenced by the Shanghai Shenggong Biological Engineering Co., Ltd. DNAMAN software (http://dnaman.software.informer.com/6.0/) was used to select specific sequences. Specific sequences of the positive clones were compared with the sequence of Nipponbare rice in the National Center for Biotechnology (NCBI) database.

RESULTS

Analysis of the AFLP polymorphic map

Twelve pairs of optimized AFLP primers amplified fragments of DNA from CK_1, CK_2, B_1, and B_2. Fourteen specific bands and 9 absent bands were identified in the mutant rice (B_1, B_2; Figure 1A-I). The AFLP amplified bands were determined by restriction enzymes and selective bases, thus, the specific bands and bands absent from B_1 and B_2 were caused by exogenous DNA. The exogenous DNA may cause changes in the recognition sequence of restriction enzymes or (and) base mutations, and deletion or insertion of nucleotide fragments may occur.

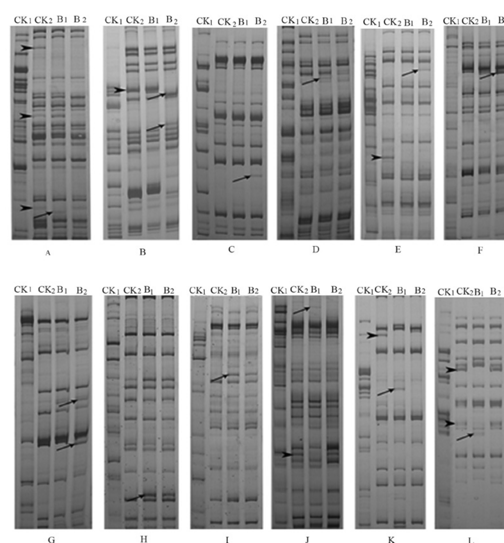

Figure 1. Amplified fragment length polymorphism (AFLP) fingerprinting of exogenous corn (*Zea mayz*; CK_1), receptor rice (*Oryza sativa*; CK_2), and two mutant F3 rice lines (B_1, B_2). Panels A to L represent results from AFLP selective amplification primer pairs P1M1, P1M2, P1M3, P2M4, P3M1, P3M5, P4M6, P4M8, P5M4, P7M1, P8M3, and P8M4, respectively. arrow, denotes specific bands; arrowhead , denotes deleted band.

Sequence analysis

Sequencing results showed 144 mutant alleles in 3791 bases (Table 2), with an overall mutation rate of 3.80%. In B_1, there were 111 mutant alleles in 2287 bases, with an overall mutation rate of 4.85%. In B_2, there were 33 mutant alleles in 1504 bases, with an overall mutation rate of 2.19%. The analysis of mutations showed that there were 43 single-base mutations, 5 double-base

mutations, 1 continuous trinucleotide mutation, 1 continuous base insertion, and 4 continuous base deletions. The frequency of single-base mutations was highest. The results demonstrate that exogenous corn DNA can induce base mutations in the genome of the receptor rice.

Table 2. Base analysis of 14 specific sequences identified in two F3 rice mutants generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Variant	Total bases (N)	Total mutant bases (N)	Single-base mutations (N)	Double-base mutations (N)	Three-sequential base mutations (N)	Multiple-sequential base mutations (N)	Mutation rate (%)
B_1	2287	111	22	3	1	4	4.85
B_2	1504	33	21	2	0	1	2.19
Total	3791	144	43	5	1	5	3.80

Tables 3 to 9 show the base mutations in 14 specific sequences and Table 10 shows the results of the recognition sequence of restriction enzymes and selective bases. There was 1 single-base mutation in the P1M1-B_1-126, P1M2-B_2-283, and P2M4-B_1-325 sequences and 2 single-base mutations in the P4M8-B_1-118 sequence (Table 3). There were 3 base mutations in the P1M3-B_2-159 sequence (1 single-base mutation and 1 double-base mutation) and 9 base mutations in the P8M3-B_1-257 sequence (4 single-base mutations, 1 double-base mutation, and 1 trinucleotide mutation; Table 4). There were 4 single-base mutations in the P3M1-B_2-378 and P5M4-B_1-313 sequences (Table 5). The mutations of the above 8 specific sequences were the non-selective base mutated into selective base. However, there were no mutations in restriction enzyme recognition sequences (Table 10). These results indicate that the 8 specific sequences were caused by non-selective bases mutating into selective base.

Table 3. Alignment results of sequences P1M1-B_1-126, P1M2-B_2-283, P2M4-B_1-325, and P4M8-B_1-118 identified in two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Mutation site	8		8		8		32	110
CK_2 (126 bp)	G	CK_2 (283 bp)	G	CK_2 (325 bp)	G	CK_2 (118 bp)	A	A
P1M1-B_1-126	A	P1M2-B_2-283	A	P2M4-B_1-325	A	P4M8-B_1-118	G	G

Sequence PxMy-B_1(B_2)-n: PxMy refers to the selective amplification primer combinations; x, y refers to the selective amplification primers 1 to 8; n refers to the number of bases of the specific sequence; CK_2 refers to the receptor rice "Nipponbare".

Table 4. Alignment results of sequences P1M3-B_2-159 and P8M3-B_1-257 identified in two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Mutation site	130-131	154		6	66	82	170	211-212	241-243
CK_2 (161 bp)	TA	C	CK_2 (254 bp)	T	C	T	G	CA	-
P1M3-B_2-159	-	T	P8M3-B_1-257	A	A	C	C	TG	CTT

- refers to a deletion; sequence PxMy-B_1(B_2)-n: PxMy refers to the selective amplification primer combinations; x, y refers to the selective amplification primers 1 to 8; n refers to the number of bases of the specific sequence; CK_2 refers to the receptor rice "Nipponbare".

Table 5. Alignment results of sequences P3M1-B_2-378 and P5M4-B_1-313 identified in two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Mutation site	6	53	209	272		7	9	22	276
CK_2 (378 bp)	T	T	C	A	CK_2 (313 bp)	C	A	A	A
P3M1-B_2-378	A	C	A	C	P5M4-B_1-313	A	G	G	G

Sequence PxMy-B_1(B_2)-n: PxMy refers to the selective amplification primer combinations; x, y refers to the selective amplification primers 1 to 8; n refers to the number of bases of the specific sequence; CK_2 refers to the receptor rice "Nipponbare".

Table 6. Alignment results of sequences P7M1-B_1-581 and P4M6-B_2-278 identified in two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Mutation site	4	75	179	296	530		1	4	153	157	219	224
CK_2 (581 bp)	T	A	C	T	T	CK_2 (278 bp)	G	T	C	A	G	C
P7M1-B_1-581	A	G	T	C	C	P4M6-B_2-278	C	C	T	T	A	T

Sequence PxMy-B_1(B_2)-n: PxMy refers to the selective amplification primer combinations; x, y refers to the selective amplification primers 1 to 8; n refers to the number of bases of the specific sequence; CK_2 refers to the receptor rice "Nipponbare".

Table 7. Alignment results of sequence P4M6-B_2-188 identified in two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Mutation site	1-2	20	50	62	85	101	112	126	154
CK_2 (188 bp)	CA	G	A	G	G	C	A	T	T
P4M6-B_2-188	TT	A	G	A	A	T	G	C	C

Sequence PxMy-B_1(B_2)-n: PxMy refers to the selective amplification primer combinations; x, y refers to the selective amplification primers 1 to 8; n refers to the number of bases of the specific sequence; CK_2 refers to the receptor rice "Nipponbare".

Table 8. Alignment results of sequences P1M2-B_2-218, P8M4-B_1-200 identified in two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Mutant site	177-184		47	133-145	150
CK_2 (210 bp)	-	CK_2 (213 bp)	C	AACCATATTTTTG	T
P1M2-B_2-218	GAGCCACT	P8M4-B_1-200	T	-	C

- refers to a deletion; sequence PxMy-B_1(B_2)-n: PxMy refers to the selective amplification primer combinations; x, y refers to the selective amplification primers 1 to 8; n refers to the number of bases of the specific sequence; CK_2 refers to the receptor rice "Nipponbare".

Table 9. Alignment results of sequence P3M5-B_1-367 identified in two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Mutation site	29	203	245-263	265-266	293-294	300-315	331	336-367
CK_2 (434 bp)	G	A	TTTTTAAAAC CAAAGTATC	TG	AC	TACCACGA TAACCGTA	G	CCCTACTCAAAACGGT TTGGTAAACCTCGAGC
P3M5-B_1-367	A	T	-	CA	GT	-	A	-

- refers to a deletion; sequence PxMy-B_1(B_2)-n: PxMy refers to the selective amplification primer combinations; x, y refers to the selective amplification primer 1 to 8; n refers to the number of bases of the specific sequence; CK_2 refers to the receptor rice "Nipponbare".

Table 10. Base mutation analysis of selective bases and restriction endonuclease recognition sequences identified in two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*; CK_1) and rice (*Oryza sativa*; CK_2).

Specific sequence	*Pst*I recognition sequence and selective bases	*Mse*I recognition sequence and selective bases
	CK_2 mutant material	CK_2 mutant material
P1M1-B1-126	5'-CTGCAGAGC→CTGCAGAAC	5'-TTGTTAA→TTGTTAA
P1M2-B2-283	5'-CTGCAGAGC→CTGCAGAAC	5'-GTGTTAA→GTGTTAA
P1M2-B2-218	5'-GTTCTGCAG→GTTCTGCAG	5'-TTAACAC→TTAACAC
P1M3-B2-159	5'-CTGCAGAAC→CTGCAGAAC	5'-CCGTTAA→CTGTTAA
P2M4-B1-325	5'-CTGCAGAGG→CTGCAGAAG	5'-ATGTTAA→ATGTTAA
P3M1-B2-378	5'-TGTCTGCAG→TGTCTGCAG	5'-TTAACTA→TTAACAA
P3M5-B1-367	5'-TGTCTGCAG→TGTCTGCAG	5'-TTAACTA→TTAACTA
P4M6-B2-188	5'-GGTCTGCAG→GGTCTGCAG	5'-CAAACTC→TTAACTC
P4M6-B2-278	5'-GTGTAGACC→CTGCAGACC	5'-GAGTTAA→GAGTTAA
P4M8-B1-118	5'-GATCTGCAG→GGTCTGCAG	5'-TTAACTT→TTAACTT
P5M4-B1-313	5'-CTGCAGCCA→CTGCAGACG	5'-ATGTTAA→ATGTTAA
P7M1-B1-581	5'-GCTCTGCAG→GCTCTGCAG	5'-TTATCAA→TTAACAA
P8M3-B1-257	5'-CCTCTGCAG→CCTCTGCAG	5'-TTAACTG→TTAACAG
P8M4-B1-200	5'-CTCCAGAGG→CTCCAGAGG	5'-ATGTTAA→ATGTTAA

*Pst*I and *Mse*I refer to two types of restriction endonucleases. Bases underlined refer to selective bases; bases in box refer to mutant base.

There were 5 single-base mutations in the P7M1-B_1-581 sequence, 6 single-base mutations in the P4M6-B_2-278 sequence (Table 6), and there were 10 base mutations in the P4M6-B_2-188 sequence (8 single-base mutations and 1 double-base mutation; Table 7). The mutations in these 3 specific sequences involved non-selective bases mutating into selective bases, and there was no mutation in selective bases (Table 10).

Eight bases were inserted into loci 177-178 of the P1M2-10-218 sequence, 13 bases were deleted from loci 133-145 in the P8M4-9-200 sequence, and 2 single-base mutations occurred in the P8M4-9-200 sequence (Table 8). There were 74 base mutations in the P3M5-9-367 sequence, including 3 single-base mutations, 2 double-base mutations, and 3 DNA fragment deletions (19 bases deleted in loci 245-263, 16 bases deleted in loci 300-315, and 32 bases deleted in loci 336-367; Table 9). There were no mutations in restriction enzyme recognition sequences and selective bases in the P1M2-10-218, P8M4-9-200, and P3M5-9-367 sequences (Table 10). These results suggest that these 3 specific sequences were caused by the deletion or insertion of nucleotide fragments.

Analysis of mutation type

One hundred and forty-four base mutations were observed in 54 loci of 14 specific bands (Table 11), including 51 base replacements in the 47 loci. The base and loci mutation frequencies were 35.42 and 87.04%, respectively. There were also 11 insertions in 2 loci, with base and loci mutation frequencies of 7.64 and 3.70%, respectively. Thus, it can be seen that deletion was the most frequent type of mutation; however, the prevalence of transition was significantly higher than that of insertion and deletion.

Types of base replacement

Table 12 shows that there were 51 base replacements, including 12 base transversions (23.53%) and 39 base transitions (76.47%). The rate of base transitions was 3.25 times higher than that of transversions. For base transversions, the number of A to T, C to A, and T to A transversions

was 3, the number of A to C transversions was 1, and the number of G to C transversions was 2. G to T, C to G, and T to G transversions were not observed in our study. These results suggest that A to T, C to A, and T to A were the major transversions. For transitions, the number of G to A, A to G, C to T, and T to C transversions was 10, 10, 9, and 10, respectively. These results demonstrate that the four types of base transition differ slightly.

Table 11. Analysis of types and frequencies of base mutations identified in 14 sequences from two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Type of base mutation	Mutant base (N)	Rate of base mutation (%)	Mutant site (N)	Rate of mutant site (%)
Substitution	51	35.42	47	87.04
Insertion	11	7.64	2	3.70
Deletion	82	56.94	5	9.26
Total	144	100	54	100

Table 12. Type and frequency of mutant base substitutions identified in 14 sequences from two F3 rice mutants (B_1, B_2) generated from crosses between exogenous corn (*Zea mays*) and rice (*Oryza sativa*).

Type of mutation	Number of bases	Frequency (%)
Transversion	12	23.53
A→C	1	1.96
A→T	3	5.88
G→C	2	3.92
G→T	0	0
C→A	3	5.88
C→G	0	0
T→A	3	5.88
T→G	0	0
Transition	39	76.47
G→A	10	19.61
A→G	10	19.61
C→T	9	17.65
T→C	10	19.61
Total	51	100

DISCUSSION

The molecular mechanism of mutations in exogenous DNA has the dual function of gene transfer and mutagenesis. Wan et al. (1992) termed this type of mutation biological mutation. In the current study, using AFLP analysis, 14 specific bands induced by exogenous corn DNA were observed in the mutant rice B_1 and B_2. Sequencing analysis demonstrated that the specific bands were caused by mutations in the restriction enzyme recognition sequence or selective bases, or the deletion or insertion of nucleotide fragments, suggesting that exogenous DNA introduction may induce genetic changes in the receptor rice. Moreover, the mutations were not only present in the 14 specific bands, but also in the remaining genome. Wang et al. (2013) hybridized *Zizania* with rice and obtained a stability variant line (RZ35). Using whole genome sequencing, they found that there were a large number of nucleotide mutations in RZ35 (Wang et al., 2013). These results are consistent with our experiments and demonstrate that wide crosses and exogenous DNA can induce biological mutation. Thus, directly introducing exogenous DNA is a viable approach to induce mutation.

In the current study, the overall mutation rate observed in the mutant rice (3.80%) was

much higher than that observed in wild rice. Luo et al. (2012) hybridized *Zizania* with rice and obtained 3 stability variant lines. Using AFLP and DNA sequencing analysis, they observed an overall mutation rate of 6.4% (Luo et al., 2012), suggesting that exogenous DNA and wide crosses could induce biological mutation.

The types of mutations observed in the current study included base transitions (the main mutation was single base transition and a small number of mutations were double base transitions), insertions, and deletions (usually occurred in a series base). We found that the prevalence of transition was significantly higher than that of insertion and deletion, suggesting that the major mutant type in F3s, induced by exogenous DNA using the pollen-tube pathway method, was base transition. Whether there was insertion of an exogenous DNA fragment requires further study.

For base replacement, the frequency of transition (76.47%) was 3.25 times higher than that of base transversion (23.53%). Using analysis of base replacement, Wang et al. (2013) found that the rate of base transition was significantly higher than that of transversion in RZ35. Using RAPD analysis of whole genomes, Deng et al. (2011) compared six mutants found in lotus with a control lotus and found the rate of base transition was 2.7 times higher than that of transversion in mutant lotus induced by ion implantation, indicating that direct introduction of exogenous DNA was similar to distant hybridization and ion implantation in biological mutation. Why base transition was the major type of base replacement is not known and whether it relates to its molecular structure is unclear. These areas are worthy of further investigation.

The transfer of exogenous corn DNA may induce mutations in rice. If the mutations occur in the functional regions of the genome, this may influence expression of this gene, leading to changes in expression, structure, and function of proteins. Additionally, phenotype variation may also occur in later generations. Ji et al. (2010) reported that exogenous corn DNA (mediated by ion beam) could change the expression of proteolytic enzymes in mutant rice seedlings, possibly as a result of variation of bases. Liang and Wu (2006) introduced a G15V point mutation into the GTPase structural domain of *OsRacD* in rice and found that activity of GTPase was obviously improved in the mutant *OsRacD* through prokaryotic expression and purification. The combination of genetic variation and phenotypic variation was of great significance and value in discovering and researching the functional gene. These will be reported in a following study.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#1304317 and #31370219), the Natural Science Foundation of Henan Province (#112300410226), and the Technical System of Rice Industry in Henan Province (#Z20120402).

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