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Mutation Breeding of a *N*-methyl-*N*-nitrosourea (MNU)-Induced Rice (*Oryza sativa* L. ssp. *Indica*) Population for the Yield Attributing Traits

Tran Dang Xuan [1,*], Truong Thi Tu Anh [1], Hoang-Dung Tran [2], Tran Dang Khanh [3,4] and Tran Dang Dat [5]

1 Graduate School for International Development and Cooperation, Hiroshima University, Hiroshima 739-8529, Japan; tuanhbp@gmail.com
2 Department of Biotechnology, NTT Institute of Hi-Technology, Nguyen Tat Thanh University, Ho Chi Minh 72820, Vietnam; thdung@ntt.edu.vn
3 Department of Genetic Engineering, Agricultural Genetics Institute, Hanoi 122300, Vietnam; tdkhanh@vaas.vn
4 Center for Expert, Vietnam National University of Agriculture, Hanoi 131000, Vietnam
5 Khai Xuan International Co. Ltd., Ha Dong District, Duong Noi Ward, LK20A-20B, Khai Xuan Building, Hanoi 152611, Vietnam; khaixuan.study@gmail.com
* Correspondence: tdxuan@hiroshima-u.ac.jp; Tel.: +81-82-424-6927

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Abstract: Difficulties in breeding new rice cultivars that have a high yield, are acceptable quality, and are tolerant to environmental stresses have been the major constraint of rice production in many developing countries, as these traits are determined by multiple genes associated with complicated and uncontrollable gene segregations.Furthermore, the gene/QTL (quantitative trait locus) introduced to the cultivar is unstable due to the interaction among the active genes, which determine the phenotypic performance, not yet been well understood or controllable. In this study, the *N*-methyl-*N*-nitrosourea (MNU)-induced mutation was applied to the heterozygote of the F_1 generation from the cross between TBR_1 (female) and KD_{18} (male parent). The phenotype and genotype of the M_2 and M_3 generations were evaluated and showed that the mutant population phenotypes, including the plant height, semi-dwarfism, amylose content, protein content, gel consistency, grain yield, and spikelet fertility, varied. Interestingly, no segregation among the genotypes in the M_2 and M_3 generations was observed, while the genotypes of the control population were either paternally inherited or indeterminable when using 28 polymorphism simple sequence repeat (SSR) markers that were identified on parental lines from 200 markers. The MNU-induced mutation caused maternal inheritance in the segregating populations, as primarily important agronomic traits were maternally succeeded from the female line TBR_1. The findings of this study indicated that, through the use of MNU, the breeding of rice cultivars with close genetic backgrounds (similarity coefficient = 0.52) could be shortened by the maternal control of important qualities, such as pest and disease resistance and high yield, thus contributing to sustainable rice production for rice farmers. Further examination of rice cultivars with a greater difference in the genetic background should be subsequently conducted.

Keywords: MNU mutation; SSR marker; maternal inheritance; rice; important agronomic trait; rice breeding; sustainable rice production

1. Introduction

Food security and sustainability have focused on rice (*Oryza sativa* L.), as it is a staple crop for more than half of the world's population. Furthermore, the demands of humans necessitate an increase in rice production despite the challenges of climate change, environmental stress, and pest and disease infestation [1]. The development of new rice cultivars with a high yield, strong resistance, acceptable quality, and wide adaptation is urgently needed [2], but this commonly takes 8–10 years or more because of the great recombination in phenotypes and genotypes required, with laborious work and a huge expenditure involved. Therefore, the lack of new rice cultivars has caused unsustainable rice production in many developing countries. It has been reported that a few traits inherited from the recurrent parent (female cultivar) exhibited cytoplasmic effects in rice, such as a low temperature [3], a low yield and width of the flag leaf [4], a low grain weight [5], a low protein content [6,7], chalkiness [8], a low cooking quality [9], and low nutrient levels [7]. A genetic investigation of the inherited effects indicated that the genetic variation in the cytoplasmic effects was low (2.41%–20.80%), whereas the maternal influence on the lysine content was greater than on the protein content and index [7].

Most agronomic traits in rice are controlled by multiple genes that show complex and quantitative inheritance [10]. Here, molecular breeding is advantageous to identify the quantitative trait loci (QTLs) associated with the desired traits, as quantitative trait locus (QTL) alleles with agronomic value have been tagged by DNA markers and introduced into elite cultivars by marker-assisted selection (MAS) [10]. To date, more than 8000 QTLs have been detected and published in the Gramene-QTL database [11]. Some effective QTLs have been cloned through map-based strategies, but the precise mapping of QTL alleles with small effects has been the principal challenge [10]. The complete sequence of the rice genome has facilitated the genetic analyses of the interesting traits [12], of which simple sequence repeats (SSRs) are currently one of the main sources of genetic markers. However, the interaction among QTLs and the corresponding genes are yet to be understood. The introgression of elite traits to target rice cultivars by molecular breeding has achieved limited success, as they have linkage-drag, where the elite QTLs tightly link with unnecessary traits. Furthermore, the influence of the genotype and its interaction with the environment on the agronomic traits has caused difficulties in rice breeding because these tightly-linked traits are paternally inherited. Moreover, the newly developed cultivars should be grown in areas which have a similar climate and growing conditions as the donor parent's (male variety), otherwise, the introgressed agronomic traits may be lost after successive generations.

Induced mutation (physical or chemical) has been documented as an impressive method to effectively improve food production in rice, sunflower, wheat, and many other crop varieties [13,14]. Previous works have assessed the efficacy of mutants and indicated that induced mutation showed intensive impacts on rice production, especially in the Asia–Pacific area [15,16]. Classical mutagenesis has been recorded in more than 3000 mutant varieties in more than 200 plant species over the past six decades [17]. Many mutant varieties have been created by radiation, as direct mutants, with gamma rays (*Brassica juncea*; *Onobrychis vicifolia*; *Musa* spp.; *Ananas comosus*; *Solanum tuberosum*), x-rays (*Nelumbo nucifera*; *Zea mays*; *Hordeum vulgare*; *Triticum aestivum*), fast neutrons (*Lotus japonicas*; *Medicago truncatula; Glycine soja*; *Hordeum vulgare; Arabidopsis thaliana*), and other radiation sources [16]. Many mutants induced with chemicals have been applied to plant mutation breeding and distributed for genetic studies (*Oryza sativa; Zea mays; Sorghum bicolor*), transgenic technologies (*Zea mays*; *Gossypium* spp.) [18], and for plant reproduction via sexual breeding (*Musa* spp.) [19]. According to the Mutant Varieties Database (https://nucleus.iaea.org/Pages/mvd.aspx), many characteristics of rice (828 cultivars) have been improved by mutation, resulting in a high yield, colored grain rice, culinary quality, disease resistance, drought tolerance, a shorter duration, a shorter height, and being slightly aromatic [20]. Among the chemical mutagens, *N*-methyl-*N*-nitrosourea (MNU) obtained biological effects from the cells since it transfers a methyl group to the oxygen and nitrogen atoms of the nucleotide bases [20]. In rice (*Oryza sativa* L.), MNU-activated mutant populations were discovered to have high-frequency mutations which were valuable for genetic approaches [21]; consequently,

numerous rice mutant varieties have been directly developed from mutated populations (RD6 and RD15 in Thailand; Zhefu 802 in China; PNR-102 and PNR-381 in India, Amaroo in Australia, and Camago 8 in Costa Rica; TNDB100 and THDB in Vietnam, Shwewatun in Myanmar, and 18 varieties in Japan) [14].

Many kinds of DNA molecular markers (RFLP, RAPD, AFLP, ISSR, SSR, SNP, DArT, and Retrotransposons) have been developed and shown to be efficient in crop improvement [22]. Out of all of them, SSR markers are feasible for identifying their derivation and for classifying important inducing mutations [23]. The uses of SSR markers are convenient and effective in breeding new rice cultivars to obtain elite important agronomic traits, such as plant height, maturity, seed shattering, amylose content, yield, and resistance to disease and environmental stresses (153 hybrid lines bred from 18 parents) [23]. In previous studies, several mutant lines developed from the MNU-induced mutation showed potential for breeding new rice cultivars with a high yield and good quality (DT84DB and DT84DB x Baothai) [24,25].

Considering all of these points, to simplify the breeding of new rice cultivars that may contribute to sustainable rice production in developing countries, the current study was conducted to evaluate the phenotypic and genotypic segregation of mutant populations generated by the MNU-induced mutation and the efficacy, for the development of improving rice cultivars.

2. Materials and Methods

2.1. Rice Materials

TBR_1 is a cultivar with a high yield, resistance to pests and diseases, and is widely cultivated in southern Vietnam. Khang dan 18 (KD_{18}) is broadly planted in the northern and central regions of Vietnam with average quality and yield, but weak resistance to pests and diseases. Both TBR_1 and KD_{18} were provided by the Agricultural Genetics Institute, Hanoi, Vietnam. In this study, TBR_1 was used as the female parent and KD_{18} was used as the male parent. Both are *Indica* subtypes, where the plant height (PH) of TBR_1 (100–110 cm) is relatively taller than that of KD_{18} (95–100 cm). The field experiment was conducted near Hiroshima University (85°E, 34°23′41″N 132°43′5″E, Higashihiroshima-shi, 270–280 m elevation; 33/25 °C day/night; humidity: 60–65%; precipitation average: 1485 mm), Higashi–Hiroshima, Hiroshima, Japan. The fertilizers, weeding, water, and pesticides were provided by conditional methods in Japan. The F_1 population, derived from TBR_1 (female) x KD_{18} (male) crossing, was treated with the MNU mutation, as described previously by Anh et al. [24] with some slight modifications. The heterozygosity of the F_1 generation was identified by SSR markers, then these F1 seeds were further soaked in 150 nM MNU for 3 hours, dried, and kept in the dark for 3 months in a hermetic condition before being stored at 4 °C. The mutated F_1 (M_1) (200 seeds) was self-pollinated to yield the mutated F_2 (M_2) population and was grown in 2016 (May–September, wet season). Subsequently, the M_2 population (200 seeds) was self-pollinated in paddy fields to provide the F_3 generation (M_3) (200 seeds) and was cultivated in 2017 (May–September, wet season). A control (without mutation) from the TBR_1 (female) x KD_{18} (male) was also used as a check for comparison. Paddy rice seeds of each M1, M2, and M3 were collected, the debris was removed, and the seeds were unhusked, dried, and stored at 5 °C in the dark until planting. The rice seedlings were transplanted by hand at 20 days after the sowing. The weeds were controlled by hand and fertilizers (N: 270 kg, P: 145 kg, K: 180 kg ha^{-1}) were applied at three stages: One week after the seedlings were transplanted, at the active tillering stage (30 days after transplantation), and at the panicle initiation (45 days after transplantation). A pesticide (Sutakuru, active compound: dinotefuran) (Mitsui Chemicals, Tokyo, Japan) was applied at 2.5 kg ha^{-1}.

30 plants from the three replications were randomly selected to evaluate the phenotypic characteristics. The plant height (PH) (cm) was calculated by using the length of the tallest tiller number from the soil surface to the tip of the panicles. The panicle length (PL) was calculated by using the length (cm) from the neck to the tip of the panicles. The grain yield (tons per ha) was recorded

from the grain yield per square meter. The spikelet fertility was calculated using the ratio of the filled grains to the total grains of each plant.

The harvest rice grains were dried with an oven to maintain the seed moisture at about 12%. The 1000 seed weight was calculated in grams. Grain quality traits, including amylose, protein, and lipid contents, were measured by a PGC Shizuoka Seiki PS-500 (version 2-12, Shizuoka Seiki Co. Ltd.; Shizuoka, Japan) [24].

2.2. Genotypic Analysis

2.2.1. DNA Extraction

The total DNA from each sample was extracted by the cetyltrimethylammonium bromide (CTAB) method with some modifications [24]. A total of 0.5 g of the young leaves of each of the individual parents, M_2, M_3, and the control (without mutation) populations, were collected at 60 days after transplantation, cut into small pieces, put into a 2.0 mL centrifuge tube with balls, and kept at −80 °C for at least two hours. These were broken up into a fine powder by a Tissue Lyser II machine (Qiagen, Germany). After that, 500 µL of hot CTAB buffer (2% CTAB, 1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl, PVP) was added into each tube and vigorously vortexed. These mixtures were incubated at 65 °C for 15 mins by a Block Bath Shaker (MyBL-100S, AS ONE Corp. Japan, Osaka, Japan) and centrifuged at 12,000 rpm for 5 mins at 4 °C. The supernatant was mixed with 250 µL of chloroform: isoamyl alcohol (24:1 ratio) and kept at room temperature for 5 mins. Then, these mixtures were centrifugated at 12,000 rpm for 5 min at 4 °C and the supernatant was transferred to a new 1.5 mL microfuge tube, supplemented with 500 µL of isopropanol, and stored at −20 °C for at least 1 hour. The supernatant was discarded after centrifugation at 12,000 rpm at 4 °C for 5 mins and the pellet was then washed with 500 µL of 70% ethanol. The pellet was dried for 15 mins at 37 °C by an Iwaki Vacuum Centrifugal Evaporator HVC 500 machine (Iwaki Co., Ltd., Tokyo, Japan) and dissolved by a TE (Tris-EDTA) buffer. The DNA concentrations were determined by a Thermo Scientific Multiskan Go, µDrop™ Plate (Thermo Fisher Scientific, Finland) and were optimized to a final concentration of 100 ng/µL with the TE buffer (10 mM Tris-HCl, pH = 8, 1 mM EDTA, pH = 8). The DNA was stored at −20 °C for genetic analysis.

2.2.2. Polymorphism Screening and Mutant Population Genotyping by SSR Markers

A set of SSR markers linked with quality traits, including amylose content, gel consistency, gelatinization temperature, protein content, and plant height, were selected from the Gramene database [26]. The SSR markers linked with a semi-dwarfism gene were chosen, as described by Liang et al. [27]. They were screened among the parents on all 12 chromosomes to privilege the polymorphism markers for each population.

The progenies from the M_2, M_3, and control (without mutation) populations were genotyped with the SSR polymorphic markers to explore their genotypic segregation. The Polymerase Chain Reaction (PCR) analysis was performed on a Thermal Cycler Gene Atlas S (ASTEC Co. Ltd; Fukuoka, Japan) [24]. The thermal program was as follows: An initial denature at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 5 min. Each PCR mixture had a final volume of 10 µL, containing 100 ng of genomic DNA, 1 × PCR buffer, 2 mM of $MgCl_2$, 100 µM of dNTPs (dideoxynucleotides), 0.5 µM of forward and reserve primer, and 1.5 U of Taq DNA Polymerase. The PCR products were separated by electrophoresis in 3% agarose in TBE 0.5× (Tris-base, Boric acid, 0.5 M EDTA) with Safeview™ classic (Applied Biological Materials Inc., Richmond, BC, Canada).

2.3. Data Analysis

The phenotypic parameters were evaluated in three replications in a completely randomized block design. The data were analyzed by the SPSS 20.0 package (SPSS Inc.; Chicago, IL, USA) and Minitab

version 16 software (Minitab Inc., Stage College, PA, USA). Normal distributions of the phenotypic traits in the parents and the M_2 and M_3 populations were calculated by descriptive statistics. The mean square value of two-way ANOVA (analysis of variance) was measured by the general linear model univariate procedure to determine the genotypic and environmental variations among the phenotypic characters [28]. During this analysis, the year was considered as the environment, and the correlation analysis among the phenotypic characters in two years (two environments) was analyzed using the Pearson correlation coefficient test [24].

3. Results

3.1. Agronomic and Crop Quality Trait Performance of the Mutant Populations

As per Table 1, the summary statistics are presented for the phenotypic traits of the parent and rice mutant populations from 2016 to 2017. Among the 13 evaluated phenotypes of the parental lines, nine characteristics of the TBR_1 (female parent) had a significantly higher value than those of the KD_{18} (male parent) in 2016, including the plant height (cm), panicle length (cm), grain length (mm), grain width (mm), grain length-to-width ratio (mm), 1000-grain weight (g), grain yield, amylose content (%), and lipid content (%), although the values of the total grain weight per plant, grain yield, and amylose content in 2017 were non-significant. In contrast, the values of the number of panicles and the grain width of KD_{18} were significantly greater than those of TBR_1. The values of the spikelet fertility and protein content in the two parent cultivars were similar between the 2016 and 2017 cropping seasons (Table 1). In the mutated population, the following phenotypes followed the recurrent parent: The number of panicles (2016), grain length (2016, 2017), grain length-to-width ratio (2016, 2017), total grain weight per plant (2017), spikelet fertility (2017), protein content (2017), and lipid content (2017), whereas the grain width (2016, 2017), total grain weight (2016), and grain yield (2016, 2017) followed the donor parent (KD_{18}). Other phenotypes were either average or greater than the values of the TBR_1 and KD_{18} (Table 1). As can be seen from Table 1, the investigated phenotypes were principally trended towards the recurrent parents, rather than the donor parents, although their values varied between the two cropping seasons in 2016 and 2017. Meanwhile, in the control population, the phenotypes were complicated and distorted the segregation of either the male or female cultivars (Table 1).

Table 1. The descriptive statistics of the phenotypic traits in the parent (M_2 and M_3) populations in 2016 (upper values) and 2017 (lower values).

Traits	Parents (Mean ± SE)		Mutated Populations	Control Populations
	Female (TBR_1)	Male (KD_{18})	Mean ± SE	Mean ± SE
PH	117.27 ± 0.50a [a]	114.20 ± 0.60b [a]	108.78 ± 0.60c [b]	113.07 ± 0.49b [b]
	117.90 ± 0.85a [a]	114.20 ± 0.60b [a]	114.80 ± 0.76b [a]	116 ± 0.43ab [a]
NP	4.90 ± 0.16b [a]	5.57 ± 0.22a [a]	4.93 ± 0.23b [b]	6.33 ± 0.26a [a]
	4.80 ± 0.00c [a]	5.57 ± 0.22b [a]	6.53 ± 0.00a [a]	5.13 ± 0.18bc [b]
PL	26.25 ± 0.29a [a]	23.19 ± 0.13c [a]	24.62 ± 0.21b [a]	23.90 ± 0.16c [a]
	26.24 ± 0.29a [a]	23.19 ± 0.13c [a]	24.27 ± 0.16b [a]	24.55 ± 0.21b [a]
GL	6.03 ± 0.02b [a]	5.53 ± 0.03c [a]	6.15 ± 0.02a [a]	5.99 ± 0.02b [a]
	6.03 ± 0.02b [a]	5.53 ± 0.03c [a]	6.14 ± 0.02a [a]	6.01 ± 0.01b [a]
GW	2.10 ± 0.02b [a]	2.18 ± 0.01a [a]	2.17 ± 0.01a [a]	2.17 ± 0.03a [a]
	2.10 ± 0.02b [a]	2.18 ± 0.01a [a]	2.13 ± 0.01ab [b]	2.17 ± 0.01ab [a]
LWR	2.88 ± 0.03a [a]	2.54 ± 0.02b [a]	2.84 ± 0.01a [b]	2.76 ± 0.07b [a]
	2.88 ± 0.03a [a]	2.54 ± 0.02b [a]	2.88 ± 0.01a [a]	2.78 ± 0.01b [a]
FG	21.60 ± 0.65a [b]	19.66 ± 0.42b [b]	18.69 ± 0.56b [b]	17.53 ± 0.84c [a]
	26.22 ± 1.16ab [a]	25.55 ± 0.91b [a]	27.91 ± 1.23a [a]	18.24 ± 0.71c [a]
SP	0.90 ± 0.01a [b]	0.89 ± 0.01a [b]	0.75 ± 0.01b [b]	0.72 ± 0.01c [a]
	0.92 ± 0.00b [a]	0.98 ± 0.01a [a]	0.90 ± 0.01b [a]	0.74 ± 0.01c [a]
KWG	19.19 ± 0.07b [a]	18.54 ± 0.06c [a]	20.01 ± 0.09a [a]	19.99 ± 0.09a [a]
	19.35 ± 0.06b [a]	18.63 ± 0.07c [a]	19.86 ± 0.08a [a]	19.31 ± 0.07b [b]

Table 1. *Cont.*

Traits	Parents (Mean ± SE)		Mutated Populations	Control Populations
	Female (TBR_1)	Male (KD_{18})	Mean ± SE	Mean ± SE
GY	7.13 ± 0.22a [b]	6.49 ± 0.14b [b]	6.17 ± 0.18b [b]	5.59 ± 0.28c [a]
	8.65 ± 0.38b [a]	7.77 ± 0.30ab [a]	9.21 ± 0.41a [a]	6.02 ± 0.23c [a]
PC *	6.22 ± 0.19ab [a]	5.62 ± 0.10b [a]	6.87 ± 0.33a [a]	6.28 ± 0.28ab [b]
	7.62 ± 2.50a [a]	6.29 ± 0.1.27a [a]	5.04 ± 0.10a [b]	7.07 ± 0.22a [a]
AC	22.72 ± 0.07c [a]	23.60 ± 0.06a [a]	23.19 ± 0.17b [a]	23.40 ± 0.15ab [a]
	22.46 ± 0.09ab [b]	23.41 ± 0.06a [b]	20.98 ± 0.93b [b]	23.07 ± 0.16a [a]
LC	9.32 ± 0.20b [a]	8.60 ± 0.16c [a]	15.10 ± 0.24a [a]	9.77 ± 0.36b [a]
	9.27 ± 0.21a [a]	8.33 ± 0.15b [a]	9.23 ± 0.13a [b]	10.14 ± 0.23a [a]

Notes: a, b, c: Different letters in the same row indicate significant differences at the $p < 0.05$ level by Fisher pairwise comparisons; *: Different letters in the same row indicate significant differences at the $p < 0.05$ level by Turkey pairwise comparisons; [a], [b]: Different letters indicate significant differences in each trait over two years (2016 and 2017) at the $p < 0.05$ level by the *t*-test; SE: Standard error; PH: Plant height; NP: Number of panicles per plant; PL: Panicle length; GL: Grain length; GW: Grain width; LWR: Grain length-to-width ratio; FG: Total grain weight per plant; SP: Spikelet fertility; KWG: 1000-grain weight; GY: Grain yield; PC: Protein content; AC: Amylose content; LC: Lipid content.

3.2. Analysis of Variance among Phenotypes

Table 2 presents the variances of the phenotype, the genotypes, and the interaction between the genotype and environment (G × E) of the parents and mutant progenies using two-way ANOVA (Analysis of the Variance). It can be seen from this table that the genotypic variances for the plant height, grain length, number of panicles, grain yield, amylose content, and lipid content were significantly different at $p < 0.001$, with total ratios of the variance of 1.62%, 55.56%, 1.04%, 1.08%, 2.2 %, and 0.16%, respectively. However, the genotypic coefficient of protein was 1.37% ($p < 0.01$), whereas the other traits were not significant (Table 2).

Table 2. The mean square values from two-way ANOVA among the phenotypic traits.

Sources	Mean Square Values		
	Genotypes	Environments	G × E
df	31	1	31
PH	20.93 ***	1255.63 ***	17.11 *
NP	2.15	42.19 ***	1.93
PL	1.93	4.82	1.42
GL	0.05 ***	0.02	0.02
GW	0.01	0.48 ***	0.01
LWR	0.02	0.76 ***	0.02
FG	47.20 ***	4467.76 ***	40.59 ***
SP	0.01	1.18 ***	0.00
KWG	0.26	13.52 ***	0.30
GY	5.14 ***	468.13 ***	4.42 ***
PC	2.36 **	167.31 ***	2.23 *
AC	0.71 ***	30.98 ***	0.60 ***
LC	2.46 ***	1578.39 ***	2.05 *

Notes: ANOVA: Analysis of the variance. *, **, ***: Indicate significance at the $p < 0.05$, 0.01, and 0.001 levels, respectively. df: Degree of freedom; G × E: The interaction between the genotype and environment, respectively; PH: Plant height; NP: Number of panicles per plant; PL: Panicle length; GL: Grain length; GW: Grain width; LWR: Grain length-to-width ratio; FG: Total grain weight per plant; SP: Spikelet fertility; KWG: 1000-grain weight; GY: Grain yield; PC: Protein content; AC: Amylose content; LC: Lipid content.

The variance from the environment for the plant height, number of panicles, grain width, grain length-to-width ration, total grain weight, grain yield, spikelet fertility, 1000-grain weight, protein content, amylose content, and lipid content were highly significant at $p < 0.001$ (Table 2) and were 99.7%, 99.16%, 98.07%, 98.00%, 97.33%, 97.06%, 96.02%, 96.00%, 95.94%, and 91.18%, respectively.

In contrast, the interaction between the genotype and the environment for the investigated agronomic traits was small. Significant interactions were found in six characteristics, including the amylose content (1.86%, $p < 0.001$), total grain weight (0.89%, $p < 0.001$), grain yield (0.92%, $p < 0.001$), plant height (1.32%, $p < 0.05$), protein content (1.30%, $p < 0.05$), and lipid content (0.13%, $p < 0.05$).

3.3. Correlations of Phenotypic Parameters

The correlations between the phenotypes of the parental lines and the mutant populations were also analyzed ($p < 0.05$ and 0.01) (Table 3). Highly significant and positive correlations were found between the grain yield (GY) and the total grain weight per plant (FG) ($r = 0.89$ and $r = 0.97$, respectively) ($p < 0.01$) in both years. A positive correlation was also detected between the grain yield (GY) and the spikelet fertility (SP) ($r = 0.32$, $p < 0.01$) in 2016. In addition, significant but negative correlations were observed between the grain width (GW) and the grain length-to-width ratio (LWR) ($r = –0.92$, $r = –0.75$, respectively) ($p < 0.01$).

An inconsistent association between the plant height (PH) and other traits was observed. In 2016, the plant height (PH) showed no correlation with the number of panicles (NP), but in 2017, the PH exerted a positive correlation with the SP ($r = 0.44$, $p < 0.01$). Negative correlations of the PH and the grain length (GL) ($r = -0.21$, $p < 0.05$) in 2016 with the grain length-to-width ratio (LWR) ($r = -0.22$, $p < 0.05$) in 2017 were found. In addition, the PH displayed an inconstant and negative correlation with the protein content (PC) in 2016 ($r = -0.22$, $p < 0.01$), but a positive correlation ($r = 0.32$, $p < 0.01$) in 2017.

The correlation between the amylose content (AC) and the other phenotypes in the mutant populationswas also analyzed (Table 2). The amylose content showed a significant positive correlation with the protein content in 2016 ($r = 0.60$, $p < 0.01$); however, a negative significant correlation was found in 2017 ($r = -0.38$, $p < 0.01$). The AC had a positive correlation with the lipid content (LC) in 2016 ($r = 0.35$, $p < 0.01$), but no correlation was found in 2017 (Table 2). Similarly, the AC showed unfavorable correlations with the LWR and the NP ($r = -0.25$ and $r = 0.22$, respectively, $p < 0.05$) in 2017. A negligible and inconsistent correlation between the PC and other traits was found. In addition, the PC exerted a high and remarkable correlation with the lipid content (LP) ($r = 0.51$, $p < 0.01$) only in 2016 (Table 3).

Table 3. The correlation among the phenotypic traits from 2016 (upper values) to 2017 (lower values).

Traits	NP	PL	GL	GW	LWR	FG	TGP	SP	THG	KWG	GY	PC	AC	LC
PH	0.17	−0.03	−0.29 **	0.09	−0.19	0.09	0.14	−0.10	0.03	0.03	0.09	−0.22 *	0.07	−0.35 **
	0.44 **	0.13	−0.14	0.15	−0.22 *	0.13	0.10	0.08	−0.11	−0.11	0.13	0.32 **	−0.19	−0.14
NP	1	0.05	−0.07	0.19	−0.17	0.12	0.11	0.04	0.11	0.11	0.12	−0.02	−0.09	−0.04
		−0.01	0.02	0.01	−0.01	0.17	0.16	0.06	0.15	0.15	0.17	0.22 *	−0.21 *	−0.09
PL		1	0.12	−0.05	0.08	−0.01	0.00	−0.02	−0.07	−0.07	−0.01	−0.02	0.00	−0.03
			−0.09	−0.14	0.06	0.17	0.16	0.01	−0.18	−0.18	0.17	−0.01	−0.13	−0.11
GL			1	−0.26 *	0.61 **	−0.10	−0.08	−0.03	−0.05	−0.05	−0.10	0.04	−0.09	0.13
				0.01	0.65 **	−0.05	−0.07	0.07	0.02	0.02	−0.05	0.02	−0.18	0.06
GW				1	−0.92 **	0.04	0.00	0.08	0.11	0.11	0.05	−0.09	0.05	−0.05
					−0.75 **	−0.07	−0.11	0.22 *	−0.07	−0.07	−0.07	0.00	0.17	−0.09
LWR					1	−0.08	−0.05	−0.07	−0.10	−0.10	−0.08	0.08	−0.09	0.09
						0.01	0.03	−0.12	0.05	0.05	0.01	0.01	−0.25 *	0.11
FG						1	0.89 **	0.31 **	−0.13	−0.13	1.00 **	−0.05	0.00	−0.16
							0.97 **	0.06	−0.16	−0.16	1.00 **	−0.07	−0.11	0.03
TGP							1	−0.16	−0.06	−0.06	0.89 **	−0.04	0.08	0.00
								−0.16	−0.17	−0.17	0.97 **	−0.08	−0.14	0.05
SP								1	−0.19	−0.19	0.32 **	−0.03	−0.17	−0.30 **
									0.05	0.05	0.06	0.01	0.18	−0.06
THG									1	1.00 **	−0.13	−0.13	−0.11	0.07
										1.00 **	−0.16	0.00	−0.06	0.00
KWG										1	−0.13	−0.13	−0.11	0.07
											−0.16	0.00	−0.06	0.00
GY											1	−0.05	0.00	−0.16
												−0.07	−0.11	0.03
PC												1	0.60 **	0.51 **
													−0.38 **	0.20
AC													1	0.35 **
														0.05

Notes: *, **: Indicates that the correlation is significant at the 0.05 and 0.01 levels, respectively; PH: Plant height; NP: Number of panicles per plant; PL: Panicle length; GL: Grain length; GW: Grain width; LWR: Grain length-to-width ratio; FG: Total grain weight per plant; TGP: Total grain per plant; SP: Spikelet fertility; THG: Weight of 200 grains; KWG: 1000-grain weight; GY: Grain yield; PC: Protein content; AC: Amylose content; LC: Lipid content.

3.4. Identification of Marker Polymorphism and Genotypic Segregation of the Phenotypic Characteristics in M_2 and M_3 Populations

To analyze the genotypes of the mutated M_2 and M_3 populations, as well as the control populations, 200 SSR markers correlated with the quality traits, plant height, and semi-dwarfism were screened on the parental cultivars to identify polymorphic markers. A total of 28 SSR markers were found to be polymorphic, which were distributed on 10 different chromosomes of the parent rice varieties TBR_1 and KD_{18}. The polymorphic SSR markers were distributed on chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, and 11, with five markers being located on chromosomes 6 and 9. Chromosomes 2 and 1 possessed four markers, three markers were found on chromosomes 1 and 4, and only one marker was observed on chromosomes 5, 7, and 8 (Table 4).

Among the 28 polymorphic SSR markers, the number of markers belonging to the amylose content, plant height, gel consistency, blast disease resistance, spikelet fertility, grain yield, brown planthopper, spikelet number, semi-dwarfism, and gelatinization temperature were subsequently 14, 8, 5, 4, 3, 2, 1, 1, and 1, respectively (Table 4). Basically, these were all nucleus-mediated agronomic traits and were inherited from the donor (male) parent. These polymorphic SSR markers were genotyped on the mutated M_2, M_3, and the control populations (Table 4). Interestingly, all of the mutant progenies carried only female alleles. None of the mutants carried male alleles (Supplementary Materials, Figures S1–S56). In contrast, the evaluation of the genotypes of the control populations based on these markers showed that the control populations carried paternal, maternal, and heterozygosity alleles (Supplementary Materials, Figures S57–S63).

Table 4. The polymorphic markers, their distribution on the chromosomes, and the correlated traits.

No	Markers	Chr.	Sequences		Mapped Location	Map Set	Correlated Traits
			Forwards	Reverses			
1	RM297	1	5′-TCTTTGGAGGCGAGCTGAG	3′-CGAAGGGTACATCTGCTTAG	132–132 cM	IRMI 2003	PC, GT
2	RM414	1	5′-TAGGGCAATTGTGCAAGTGG	3′-TTGGGAATTGGGTAGGACAG	191.1–191.1 cM	Cornell SSR 2001	GC, PH
3	RM207	2	5′-CCATTCGTGAGAAGATCTGA	3′-CACCTCATCCTCGTAACGCC	191.2–191.2 cM	Cornell SSR 2001	BDR, PH
4	RM213	2	5′-ATCTGTTTGCAGGGGACAAG	3′-AGGTCTAGACGATGTCGTGA	186.4–186.4 cM	Cornell SSR 2001	BDR, AC
5	RM6	2	5′-GTCCCCTCCACCCAATTC	3′-TCGTCTACTGTTGGCTGCAC	145.1–145.1 cM	CIAT SSR 2006	PC
6	RM318	2	5′-GTACGGAAAACATGGTAGGAAG	3′-TCGAGGGAAGGATCTGGTC	145.4–145.4 cM	CIAT SSR 2006	PC
7	RM7	3	5′-TTCGCCATGAAGTCTCTCG	3′-CCTCCCATCATTTCGTTGTT	48.6–48.6 cM	CIAT SSR 2006	SN, AC
8	RM251	3	5′-GAATGGCAATGGCGCTAG	3′-ATGCGGTTCAAGATTCGATC	79.1–79.1 cM	Cornell SSR 2001	AC
9	RM3392	3	5′-GTCCAATGATTCGTTCCCAC	3′-CTTCACCGTTCACCAATTCC	18.7–18.7 cM	CIAT SSR 2006	AC
10	RM273	4	5′-GAAGCCGTCGTGAAGTTACC	3′-GTTTCTACCTGATCGCGAC	116.8–116.8 cM	CIAT SSR 2006	GC
11	RM307	4	5′-GTACTACCGACCTACCGTTCAC	3′-CTGCTATGCATGAACTGCTC	0–0 cM	Cornell SSR 2001	BDR, PH
12	SSR371*	5	5′-TGCGATGAGATTACGAGACC	3′-ACAGATTATTTGCTCACGCTAT	0.5-2.0 cM	AC104708	Semi-dwarfism
13	RM508	6	5′-GGATAGATCATGTGTGGGGG	3′-ACCCGTGAACCACAAAGAAC	0–0 cM	Cornell SSR 2001	AC
14	RM587	6	5′-ACGCGAACAAATTAACAGCC	3′-CTTTGCTACCAGTAGATCCAGC	10.7–10.7 cM	Cornell SSR 2001	AC
15	RM589	6	5′-ATCATGGTCGGTGGCTTAAC	3′-CAGGTTCCAACCAGACACTG	3.2–3.2 cM	Cornell SSR 2001	AC, GC
16	RM527	6	5′-GGCTCGATCTAGAAAATCCG	3′-TTGCACAGGTTGCGATAGAG	61.2–61.2 cM	Cornel SSR 2001	GC
17	RM584	6	5′-AGAAAGTGGATCAGGAAGGC	3′-GATCCTGCAGGTAACCACAC	26.2–26.2 cM	Cornel SSR 2001	AC, GC
18	RM432	7	5′-TTCTGTCTCACGCTGGATTG	3′-AGCTGCGTACGTGATGAATG	43.5–43.5 cM	Cornel SSR 2001	AC, GY
19	RM149	8	5′-GCTGACCAACGAACCTAGGCCG	3′-GTTGGAAGCCTTTCCTCGTAACACG	122.1–122.1 cM	CIAT SSR 2006	BPR, PH, SP
20	RM219	9	5′-CGTCGGATGATGTAAAGCCT	3′-CATATCGGCATTCGCCTG	11.7–11.7 cM	Cornell SSR 2001	PH, GY, AC
21	RM107	9	5′-AGATCGAAGCATCGCGCCCGAG	3′-ACTGCGTCCTCTGGGTTCCCGG	82.4–82.4 cM	Cornell SSR 2001	AC
22	RM434	9	5′-GCCTCATCCCTCTAACCCTC	3′-CAAGAAAGATCAGTGCGTGG	57.7–57.7 cM	Cornell SSR 2001	AC
23	RM5688	9	5′-GCAGTGTCCAACCATCTGTG	3′-ATCTGGTCACCCTTTGCTTG	30.8–30.8 cM	IRMI 2003	AC
24	RM201	9	5′-CTCGTTTATTACCTACAGTACC	3′-CTACCTCCTTTCTAGACCGATA	81.2–81.2 cM	Cornell SSR 2001	BDR, GY, PH
25	RM229	11	5′-CACTCACACGAACGACTGAC	3′-CGCAGGTTCTTGTGAAATGT	77.8–77.8 cM	Cornell SSR 2001	PH, SP
26	RM202	11	5′-CAGATTGGAGATGAAGTCCTCC	3′-CCAGCAAGCATGTCAATGTA	42.1–42.1 cM	CIAT SSR 2006	SP, PH
27	RM206	11	5′-CCCATGCGTTTAACTATTCT	3′-CGTTCCATCGATCCGTATGG	104.2–104.2 cM	CIAT SSR 2006	BPR, BDR, PH, SP
28	RM287	11	5′-TTCCCTGTTAAGAGAGAAATC	3′-GTGTATTTGGTGAAAGCAAC	68.6–68.6 cM	Cornell SSR 2001	AC

Notes: Chr.: Chromosome; cM: Centimorgan; PC: Protein content; AC: Amylose content; PH: Plant height; GY: Grain yield; SP: Spikelet fertility; GC: Gel consistency; BDR: Blast disease resistance; GT: Gelatinization temperature; BPR: Brown planthopper resistance; SN: Spikelet number, sources: [26,27].

4. Discussion

For the improved genotype development, it is important to evaluate the phenotypic performance of the new cultivars in various conditional environments (either in different locations during the same season or in different seasons/years in the same location) [29]. To identify the genetic stability of a trait in a mutant population, the evaluation of the genotype of the modified traits, using molecular markers, should be conducted after phenotypic evaluation [30]. Furthermore, an induced mutation has more advantages for the variation of a few elite traits than conventional breeding [14].

The assessment of the phenotype of the parents indicated that the parental lines TBR_1 and KD_{18} were suitable for studying a wide range of diversity in the selected traits. The current study found that the parents presented stability, whereas the mutant progenies expressed the variations in two years (2016 and 2017). It was indicated that the variation may be due to the environmental influence on most phenotypic characteristics associated with genotypes [31,32]. However, due to an inconsistency between the parents and mutant progenies, a possible explanation for this variation may be that the mutation affected the rice materials and led to variations in phenotype.

The traits, including the plant height, grain length, total grain weight number per plant, grain yield, and the content of protein, amylose, and lipids, were reported to be sensitively affected by the environment [31]. Additionally, the evaluation of the interaction between the genotype and the environment was important for the improvement of agronomic traits in crops [33,34]. In this study, slight and inconsistent correlations between the phenotypic traits of mutants were identified in the two years (Table 3), which might be due to the interaction between the genotypes and their environments [32], as well as influences of the environment, such as the climate and soil [32,35]. In a previous study on transfer DNA (T-DNA) insertional mutagenesis, Lo et al. [35] stated that it was necessary to screen the developed rice mutants in a favorable environment. In MNU-induced mutant research, Suzuki et al. [21] observed that visible mutant phenotypes were segregated and numerous mutants had multiple abnormalities, out of which each phenotype was segregated independently. The current study strengthened the evidence of MNU-induced mutation and was associated with phenotypic variations. Several lines of evidence suggest that MNU, a strong chemical mutagen, can change the genomic base-pair constitution [17,21] that leads to phenotype change, as the phenotype is a result of the interaction between the genotype and the environment [36].

Polymorphism molecular markers, as an effective tool in molecular breeding, were used to measure the genetic diversity in this study [37]. The low polymorphic markers on TBR_1/KD_{18} may be due to their close relationship. The evaluation of the mutant genotypes in the two generations by polymorphic markers showed that all of the mutants possessed female parent alleles without male parent alleles. The mutants completely inherited different traits from the female parent (TBR_1) without segregation (Supplementary Materials, Figures S1–S56). This finding showed non-Mendelian inheritance (Supplementary Materials, Figures S1–S56), whilst the genotypes of the control population were uncontrollable (Supplementary Materials, Figures S57–S63). Non-Mendelian inheritance has been documented in cultivated rice (*Oryza sativa*) and wild rice (*O. rufipogon* [37–41], *O. sativa* × *O. glumaepatula* [42], *Indica* × *Japonica* [43], *O. sativa* × *O. longistaminata* [44], and *Indica* × *Javanica* [45,46]). A possible explanation for this may be due to the genetic distances of the parents [37,44,47]. The importance of genetic distance has been noted, as it creates unpredictable recombination that leads to segregation. However, in a previous study, we observed that the high genetic similarity of the female and male parents (TBR_1 and KD_{18}) was identified, with a similarity coefficient of 0.52 (Supplementary Materials, Table S1) [24]. Therefore, lesser recombination between the parent cultivars can be made in comparison to rice cultivars with a greater difference in their genetic background. Furthermore, the literature indicates that maternal inheritance usually occurs in mitochondrial and chloroplast genes [48,49]. In this study, the use of MNU mutation was successfully conducted on the maternal control of important agronomic traits (Tables 1 and 2), which were nuclear-mediated to reduce the breeding time. However, the further examination of cultivars with

a greater difference in their genetic background should be conducted to affirm the success of MNU mutation on rice breeding.

In our previous research, the effectiveness of MNU-induced mutation was demonstrated on rice genotypes [24]. As an immensely strong chemical mutagen [17], MNU resulted in a higher frequency mutation than ethyl methanesulfonate (EMS) [21,50] and created high-density mutations with a random distribution via the radiation of a single nucleotide [51]. The MNU-induced mutation altered nucleotides that caused a point mutation [17]. In addition, the ratio of amino acids was increased, a total of 360 variations were detected in the protein coding regions of the genome, and phenotypic characterization of the mutants showed abnormal levels [21].

In crops including rice, it commonly takes many years and may require up to 20 cropping cycles and heavy laborious work to breed a new cultivar with desirable agronomic characteristics. Therefore, shortening the breeding time is an essential requirement. This study demonstrated the role of MNU-induced mutation and supports the fact that conventional mutation by MNU can reduce the breeding time in rice cultivars with a close genetic background. The MNU-mutated populations, M_2 and M_3, expressed the phenotypes and genotypes that correlated to the MNU effects. Therefore, a putative gene linkage with an unknown mechanism may be induced from MNU mutation, to eliminate the complete segregation of genotypes in the M_2 and M_3 populations of TBR_1 and KD_{18} cultivars. The MNU-induced mutation probably generated a novel maternal inheritance of important agronomic traits in rice, such as quality, plant height, protein content, and pest and disease resistance, so further elaboration on the subsequent generations should be conducted to ensure the stability of the maternal inheritance. In addition, the unknown mechanism of this putative gene linkage needs clarification and examination in different populations of rice cultivars with a greater difference in their genetic background.

5. Conclusions

This study revealed that, although the phenotypic performances of both the mutant and control populations were variable, few important agronomic traits were maternally inherited in the two rice cultivar, TBR_1 and KD_{18}. The experimental findings of this study confirmed the application of MNU, in that it may assist in more effective mutation breeding for the development of various traits in new rice genotypes, which is not possible through conventional breeding. However, the efficacy of MNU mutation on different rice cultivars with a diverse genetic background requires further investigation. The search for the presence of genes related to the novel maternal inheritance should be targeted in continuous research. More tests of the MNU mutation on rice cultivars with a greater difference in their genetic background should be examined to confirm the efficacy of this induced mutation on maternal inheritance in rice breeding. It thus contributes to the sustainability of rice cultivation in many developing countries.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/11/4/1062/s1, Table S1, Figures S1–S63.

Author Contributions: T.D.X. and T.D.D. conveyed the idea of this research; T.T.T.A. conducted the experiments and wrote the manuscript under the supervision of T.D.X.; T.D.K. and T.D.D. created the mutant materials; T.T.T.A. analyzed the research data; T.D.X., H.-D.T., and T.D.K. corrected the manuscript; T.D.X. revised and approved the final version.

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