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Uniparental Inheritance of Salinity Tolerance and Beneficial Phytochemicals in Rice

Can Thu Huong [1], Truong Thi Tu Anh [2], Tran Dang Dat [3], Tran Dang Khanh [4] and Tran Dang Xuan [1,*]

1 Graduated School for Advanced Science and Engineering, Hiroshima University, 1-5-1 Kagamiyama, Higashihiroshima 739-8529, Japan; cth1412@gmail.com
2 Division of Biotechnology, Institute of Agricultural Science for Southern Vietnam, 121 Nguyen, Binh Khiem Street, District 1, Ho Chi Minh City 71058, Vietnam; tuanhbp@gmail.com
3 Khai Xuan International Co. Ltd., Ha Dong District, Duong Noi Ward, LK20A-20B, Khai Xuan Building, Hanoi 152611, Vietnam; khaixuan.study@gmail.com
4 Department of Genetic Engineering, Agricultural Genetics Institute, Hanoi 122300, Vietnam; tdkhanh@vaas.vn
* Correspondence: tdxuan@hiroshima-u.ac.jp; Tel./Fax: +81-82-424-6927

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Abstract: Salinity stress is one of the most problematic constraints to significantly reduce rice productivity. The *Saltol* QTL (quantitative trait locus) has been known as one among many principal genes/QTLs responsible for salinity tolerance in rice. However, the introgression of the *Saltol* QTL from the donor (male) into the recipient (female) cultivars induces great recessions from the progeny generation, which results in heavy fieldwork and greater cost and time required for breeding. In this study, the F_1 generation of the cross TBR1 (female cultivar, salinity tolerant) × KD18 (male cultivar, salinity susceptible) was preliminarily treated with N-methyl-N-nitrosourea (MNU) to induce the mutants M_1. Results on physiological traits show that all the M_2 (self-pollinated from M1) and M_3 (self-pollinated from M2) individuals obtain salinity tolerant levels as the recurrent TBR1. Twelve SSR (simple sequence repeat) markers involved in the *Saltol* QTL (RM493, RM562, RM10694, RM10720, RM10793, RM10852, RM13197, RM201, RM149, RM508, RM587, and RM589) and other markers related to yield-contributing traits and disease resistance, as well as water and nitrogen use, have efficacy that is polymorphic. The phenotype and genotype analyses indicate that the salinity tolerant *Saltol* QTL, growth parameter, grain yield and quality, pest resistance, water and nitrogen use efficacy, and beneficial phytochemicals including antioxidants, momilactone A (MA) and momilactone B (MB) are uniparentally inherited from the recurrent (female) TBR1 cultivar and stabilized in the M_2 and M_3 generations. Further MNU applications should be examined to induce the uniparental inheritance of other salinity tolerant genes such as *OsCPK*17, *OsRMC*, *OsNHX*1, *OsHKT*1;5 to target rice cultivars. However, the mechanism of inducing this novel uniparental inheritance for salinity tolerance by MNU application needs elaboration.

Keywords: rice; salinity tolerance; MNU treatment; uniparental inheritance; QTL; gene; climate change; momilactone A; momilactone B; antioxidants

1. Introduction

Salt is one of the main causes of land degradation worldwide with approximately 2000 million ha affected land being recorded every year, according to a study by Economics of Salt-Induced Land Degradation and Restoration (unu.edu/media-relations/releases). Salinity stress can reduce 70% yield loss of wheat, maize, rice and barley and the total cost of such loss in crop productivity can reach

US $12 billion per year globally [1]. Half of the world population consumes rice (*Oryza sativa* and *Oryza glaberrima*) as a staple food [1]. By 2030, rice production needs to increase by 25% to feed the global population, however, rice growth and productivity is threatened by abiotic stresses including cold, drought, heat, flood, and salt [2]. Rice is one of the most salt sensitive crops with a threshhod of 3 dSm^{-1} for most currently cultivated varieties [3]. At EC (electrical conductivity of its saturation extract) of 7.2 dSm^{-1}, 50% rice yield loss was recorded [4]. Besides the effects from climate change, the dam construction on the main stream of important rivers such as the Mekong River in Southeast Asia has caused severe drought in the lower basin which induces the intrusion of sea water into the coastal deltas, resulting in serious reduction of rice yield [5]. Therefore, the breeding of rice cultivars tolerant to salinity is required.

The salinity tolerance of rice has not been the priority in the breeding program until recent decades, where climate change including salinity stress is causing severe damage to rice cultivation. Different from important agronomic traits such as growth, yield, quality, and pest tolerance, few genes/QTLs (quantitative trait locus) responsible for salinity tolerance have been discovered so far [6]. By conventional breeding, at least 10 years and huge cost (US $50–900 million) are required to develop salinity tolerant varieties [1]. Marker assisted selection (MAS) can reduce the breeding time to 3–6 years, however, it is difficult to identify all genetic markers relevant to QTLs/genes tolerant to salinity in rice. Among them, the *Saltol* QTL has been discovered as a principal QTL responsible for salinity tolerance in rice [7–10]. The introduction of the *Saltol* QTL to target rice cultivars have been extensively conducted [11], however, it causes great secessions from the F_2 generation, which requires many cropping seasons (8–10 years), including both cross and backcross to stabilize the salinity tolerant trait [11,12]. The key genes involved in salinity tolerance are *OsCPK*17, *OsRMC*, *OsNHX*1, *OsHKT*1;5, and *Sal*T [6], in which *OsCPK*17 increases a transient in cytosolic Ca^{2+} under salinity stress [6]; this gene also occurs with four QTLs linked to ion homoostasis [13]. *OsRMS* is a jasmonic acid-induced DUF26 protein and is upregulated at the transcript level by high salinity [14]. The two other principal genes, *OsHKT*1;5 and *OSNHX*1, are involved in ion homoeostasis to adjust osmotic pressure by keeping Na^+ away from the cytosol [15]. *OsHKT*1;5 is responsible for retreiving Na^+ from the xylem sap to reduce Na^+ load in shoots and can balance low Na^+/K^+ shoot ratios in rice plants in salinity stress [13,16]. *OsNHX*1 encodes a vacuolar $(Na^+, K^+)H^+$ antiporter placed in the tonoplast to allow efficient compartment of Na^+ in the vacuole [17]. In addition, *Sal*T in the *Saltol* QTL, which is localized in chromosome 1, is the first isolated and characterized key gene from the roots of salinity tolerant rice [7]. This gene is regulated by abscisic acid (ABA)-dependent and ABA-independent pathways and it controls the expression with the production of osmoprotectants such as trehalose and proline [6].

By cDNA microarray and RNA gel-blot analyses, 57 stress-inducible genes were found in rice; among them, 36, 62, and 43 genes were induced by cold, drought and ABA, respectively [14]. Except for the five key genes *OsCPK*17, *OsRMC*, *OsNHX*1, *OsHKT*1;5, and *Sal*T, many candidate genes might be also involved in salinity as well as other environmental stresses, although their functions still remain unknown. To date, all QTLs/genes determining important agronomic traits such as yield, quality, growth, pest and environmental tolerance are localized in the nucleus, while a few traits inherited from the reccurent parent (female cultivar) exhibited cytoplasmic effects in rice, including low temperature [18], a low yield and width of the flag leaf [19], a low grain weight [20], a low protein content [21,22], chalkiness [23], a low cooking quality [24], and low nutrient levels [25]. The genetic variation in the cytoplasmic effects was only 2.41%–20.80%, whilst the maternal influence on the lysine content was greater than that on the protein content and index [24].

In rice as well as other cereals, the environmental stress tolerant traits do not commonly associate with important agronomic traits, including yield and quality [12]. Therefore, conventional breeding results in great recombination and recessions of phenotypes and genotypes; laborious work and huge expenditure are required to select rice lines which have acceptable yield and quality but are tolerant to pest and environmental stresses [12,25]. For instance, FL478 is a salt tolerant recombinant inbred line of IR66946-3R-178-11 developed from Pokkali (salinity tolerant) and IR29 (salinity susceptible),

and it shows low quality parameters as compared with common commercial rice [11]. Our laboratory observed that >97% kernel color of the FL478 was similar to that of Pokkali (Supplementary Table S1). In addition, the use of DNA molecular markers such as RFLP, RAPD, AFLP, ISSR, SSR, SNP, DarT and Retrotransposons are useful to reduce the time for selection, but they can not exhibit all QTLs/genes interactions involved in the selected traits [12,25].

Our group has developed a protocol using N-methyl-N-nitrosourea (MNU) to treat rice seeds at low concentrations for three months prior to germination [12,25]. The MNU application induced uniparental inheritance of the yield attributing traits, including plant height, semi-dwarfism, amylose content, protein content, gel consistency, grain yield, and spikelet fertility [12,25]. In this study, we continuously examine whether the salinity tolerant *Saltol* QTL can also be uniparentally inherited by MNU treatment. The influence of the uniparental inheritance on induction of beneficial phytochemicals including total phenols and flavonoids, antioxidant activities, and momilactones A and B in the offspring lines were also evaluated.

2. Materials and Methods

2.1. Rice Materials and Salinity Treatment

TBR1 and KD18 are commercial rice in Vietnam. Both are *Indica* subtypes, where TBR1 performs a higher yield, protein content and lipid content compared to KD18. TBR1 is resistant and KD18 is susceptible to pests and diseases. In this study, TBR1 was used as the recurrent (female) cultivar, whilst KD18 was the male variety. They were provided by the Agricultural Genetics Institute, Hanoi, Vietnam. The field experiment was conducted near Hiroshima University, Higashi–Hiroshima, Hiroshima, Japan (270–280 m elevation; 33/25 °C day/night; humidity: 60%–65%; precipitation average: 1485 mm). The fertilizers, weeding, watering, and pesticides were provided by conventional methods in Japan. The original F_1 rice seeds (total 300 seeds) were obtained by crossing TBR1 (female) and KD18 (male) cultivars. Subsequently, the F_1 seeds were treated with the MNU as described previously [12,25] to induce the first mutated generation M_1 (200 seeds). The M_1 seeds were kept in the dark for 3 months in a hermetic condition and stored at 4 °C before self-pollinating in a paddy field to obtain the second generation (M_2) seeds. The M_2 population (200 seeds) was continuously self-pollinated in rice fields to provide the third generation (M_3). After gemination, TBR1, KD18, F_1, F_2 (self-pollinated from F_1), M_2, and M_3 seeds were grown in a 0.5% agarose media supplied by Yoshida nutrient and placed in a plant growth chamber (28 °C day: 25 °C night; 12 h light: 12 h dark). Salinity was applied after five days of transplanting with a concentration of 12 dSm^{-1} NaCl to evaluate the salinity tolerant of the parental cultivars and progenies. The treatment without NaCl was considered as control (0 dSm^{-1}). During treatment, EC and pH (5.5) were checked and maintained daily.

2.2. Physiological Analysis of Salt Tolerance

After 21 days of treatment, salinity tolerant rice materials were scored by a standard evaluation score (SES) [26]. Fifty plants from the five replications were randomly selected to evaluate the phenotypic characteristics. Survivability was determined by percentage of survived plants. Root length and plant height of rice samples were measured in milimeters. Rice plants were weighed twice. Fresh weight was measured right after treatment and dry weight was determined after drying in hot air oven for 5 days at 40 °C.

2.3. Total Phenols, Total Flavonoids, and Antioxidant Activities

The roots, stems, and shoots of rice seedlings were harvested and transferred directly to laboratory. They were cleaned by tap water and rinsed many times with distilled water. After drying for 5 days at 40 °C, the mixture of roots, stems, and shoots of rice samples were ground into powder. This powder was then extracted by methanol after 3 days using a magnetic stirrer. After that, the extract was separated by hexane and finally dried by evaporator at 50 °C. The obtained powder was kept in methanol at

4 °C in the dark for further measurements. Total phenolic content (TPC) and ABTS•+ decolorization measurement were conducted following a method described by Quan et al. [27]. Total flavonoid content (TFC) was estimated as detailed in Xuan et al. [28]. 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and nitric oxide (NO) scavenging assays were evaluated by a method described in Govindarajan et al. [29]. The antioxidant activity was calculated by the following formula:

$$\text{Antioxidant activity (\%)} = (1 - A_{sample}/A_{control}) \times 100 \quad (1)$$

where A_{sample} is the absorbance of reaction with sample and $A_{control}$ is the absorbance of reaction with pure methanol. IC_{50} value is determined as the essential concentration to obtain 50% radical reduction.

2.4. Identification and Quantification of Momilactones A and B

Momilactone A (MA) and momilactone B (MB) were identified and quantified by UPLC-ESI-MS following a protocol described in Quan et al. [30] using MA and MB purified from rice husk as the standards. The quantification of each momilactone was measured using a linear model through peak areas and retention times. Detection and quantitation limits of MA and MB were 0.68 and 2.05 ng/mL and 0.27 and 0.83 ng/mL, respectively.

2.5. Genetic Analysis

The total DNA in rice samples was extracted by cetyl-trimethyl ammonium bromide (CTAB) method [12]. Before applying for polymerase chain reaction (PCR), 0.8% agarose gel electrophoresis was used to check the quality of DNA. The DNA was amplified using a Thermal Cycler Gen Atlas S machine [12]. The amplification products were resolved in 3% agarose gel in tris borate EDTA (TBE) buffer (0.5 X) and 2.5 µl of safe view under room temperature at a constant voltage of 50 volts for 75 min. After running, the gel was visualized by AMZ System Science limited STAGE system ECX-F15M (Vilber Lourmat, Eberhardzell, Germany).

A total of fifty SSR markers which were reported previously to be involved in growth parameters, yield, pest resistance, and the *Saltol* QTL (salinity tolerant) [7–10,12,31–45] were selected to evaluate the differences among genotypes of the recurrent TBR1 and male KD18 cultivars (Supplementary Figure S3). These markers are distributed throughout 12 chromosomes of the rice genome (Supplementary Table S2). Twenty-one markers including RM 493, RM 518, RM 562, RM 1233, RM 10694, RM 10720, RM 10793, RM 10852, RM 13197, RM 149, RM 201, RM 202, RM 206, RM 207, RM 213, RM 219, RM 229, RM 432, RM 508, RM 587, and RM 589 are polymorphic (Supplementary Table S2), of which, RM 493, RM 562, RM 10694, RM 10720, RM 10793, RM 10852 are closely associated with the *Saltol* QTL located on chromosome 1. This QTL is identified for shoot Na^+ concentration, shoot and root K^+ concentrations, and shoot and root Na-K ratio [7]. The marker RM 13197 is located on chromosome 2, which is influenced by seedling plant height, seedling survival, leaf chlorophyll content, and root K^+ concentration [7]. Markers RM 508, RM 587, and RM 589, which are associated with leaf diameter and root length, are located on chromosome 6 [44]. Besides these, markers relevant to other *Saltol* QTLs, such as plumule (shoot) fresh weight and root Na–K ratio, are RM 149 (chromosome 8) and RM 201 (chromosome 9), respectively [9,10]. Other polymorphic markers were identified relating to growth parameter, grain yield and quality, and pest resistance, as well as water and nitrogen use efficiency [12,41,43,45]. These polymorphic markers therefore were used to identify F_2, M_2 and M_3 genotypes.

2.6. Data Analysis

Data were statistically analysed by analysis of variance (ANOVA). A linear model was used to calculate correlation between parental and progeny data. Reduction proportions of agronomical and chemical data were calculated by following formula:

$$\% \text{ reduction} = (P_{sample} - P_{control})/P_{sample} \times 100 \quad (2)$$

where P_{sample} is value of sample growing in salt concentration 12 dSm^{-1} and $P_{control}$ is the value of sample in non-salinity.

3. Results

3.1. Salt Tolerance, Agronomical, and Phytochemical Performances of Rice Population

The results in Table 1 show that phenotypic performances among F_1 and F_2, and M_2 and M_3 are not remarkably different. In control conditions, no significant difference was observed in injury score, survivability, and growth parameters among TBR1, KD18, F_1, F_2, M_2 and M_3. In salinity stress condition, the injury score and survivability of the M_2 and M_3 were not significantly different from the recurrent parent TBR1. On the contrary, those of the F_1 and F_2 were between the values of the parents. The other growth parameters of the M_2 and M_3 including root length, shoot length, fresh weight, and dry weight were neither markedly different from TBR1 nor less affected from salinity than KD18 (Table 1), as they showed greater performances than those of the parents. In the control population (F_1 and F_2), these values were between the values of parental cultivars. Findings of Table 1 indicate that the TBR1 cultivar was salinity tolerant whilst KD18 was salinity susceptible. The response of M_2 and M_3 against salinity was as tolerant as that of the recurrent parent TBR1, thus, it was concluded that the M_2 and M_3 are also salinity tolerant. Therefore, the salinity tolerant characteristic of M_2 is uniparentally inherited from TBR1. To date, all reports in literature, such as Mishra et al. [46] and Mohammadi et al. [47], reported that the salinity tolerance trait in rice is polygenic, principally inherited from father cultivar following the Mendelian rules. However, in contrast, findings in Table 1 show that the treatment of MNU can uniparentally control the salinity tolerant *Saltol* QTL in rice, conversely different from all researches in literature.

Table 1. Phenotypic responses of examined rice in salinity stress.

NC	Samples	Injury Score	SUR (%)	RL (mm)	SL (mm)	FW (mg)	DW (mg)
Control	TBR1	1.0 ± 0.0 a	100.0 ± 0.0 a	58.4 ± 1.5 ab	188.7 ± 4.0 a	83.0 ± 3.0 ab	25.0 ± 1.0 ab
	KD18	1.0 ± 0.0 a	100.0 ± 0.0 a	61.1 ± 2.7 ab	180.7 ± 1.8 ab	85.0 ± 5.0 ab	25.0 ± 1.0 ab
	F_1	1.0 ± 0.0 a	100.0 ± 0.0 a	61.1 ± 1.8 ab	181.5 ± 2.5 ab	86.0 ± 3.0 ab	25.0 ± 1.0 ab
	F_2	1.0 ± 0.0 a	100.0 ± 0.0 a	60.9 ± 2.3 ab	184.2 ± 1.5 ab	82.0 ± 1.0 ab	25.0 ± 0.0 ab
	M_2	1.0 ± 0.0 a	100.0 ± 0.0 a	60.9 ± 1.0 ab	187.6 ± 4.6 a	97.0 ± 4.0 a	27.0 ± 1.0 a
	M_3	1.0 ± 0.0 a	100.0 ± 0.0 a	62.7 ± 1.5 ab	185.6 ± 2.3 ab	92.0 ± 3.0 a	25.0 ± 1.0 ab
Stress	TBR1	5.9 ± 0.2 b	67.0 ± 1.5 b	63.1 ± 3.0 ab	160.0 ± 4.4 b	74.0 ± 2.0 bc	21.0 ± 1.0 bc
	KD18	7.2 ± 0.1 c	32.0 ± 2.5 c	52.7 ± 2.4 b	136.8 ± 4.7 d	60.0 ± 5.0 c	19.0 ± 1.0 c
	F_1	6.5 ± 0.2 bc	47.5 ± 2.1 bc	60.7 ± 1.7 ab	151.9 ± 4.2 c	68.2 ± 4.0 bc	20.0 ± 1.0 bc
	F_2	6.8 ± 0.3 c	50.8 ± 1.5 bc	60.5 ± 4.5 ab	155.6 ± 4.5 c	70.7 ± 4.0 bc	19.0 ± 2.0 c
	M_2	6.0 ± 0.2 b	59.0 ± 1.0 b	68.0 ± 1.4 a	165.7 ± 4.0 b	86.0 ± 4.0 bc	24.0 ± 1.0 ab
	M_3	6.0 ± 0.5 b	61.5 ± 2.3 b	65.1 ± 3.5 a	167.5 ± 5.2 b	88.6 ± 3.0 b	24.0 ± 0.0 ab

Values are means ± SE (standard errors); NC: Nutrition condition, SUR: survivability, RL: Root length, SL: Shoot length, FW: Fresh weight, DW: Dry weight; Mean with same letters in a column is not significantly different ($p < 0.05$).

Table 2 shows the changes of chemical components including TPC, TFC, and contents of MA and MB, and antioxidant activities (DPPH, ABTS, and NO levels). In the untreated conditions, the phytochemical contents and antioxidant activities vary among TBR1, KD18, F_1, F_2, M_2 and M_3, whilst no trace of MB was detected. Similar with phenotypic responses, no significant disparity was recorded in chemical compositions between F_1 and F_2, and M_2 and M_3. However, in the salinity stress condition,

antioxidant activities of TBR1, F_1, F_2, M_2 and M_3 were both significantly stronger than that of KD18, of which F_1 and F_2 show weaker antioxidant properties than TBR1.

Table 2. Changes in phytochemical properties of examined rice in salt stress.

NC	Samples	TPC (mg GAE g^{-1} DW)	TFC (mg RE g^{-1} DW)	DPPH (IC_{50} mg/mL)	ABTS (IC_{50} mg/mL)	NO (IC_{50} mg/mL)	MA (ng/g)	MB (ng/g)
Control	TBR1	1.6 ± 0.1 b	0.3 ± 0.0 d	1.5 ± 0.1 b	1.8 ± 0.0 c	0.9 ± 0.0 ab	59.9 ± 1.3 c	Nd
	KD18	0.8 ± 0.2 c	0.4 ± 0.0 cd	2.4 ± 0.1 d	2.1 ± 0.0 d	1.8 ± 0.1 d	76.5 ± 2.1 b	Nd
	F_1	1.6 ± 0.2 b	0.3 ± 0.1 d	1.9 ± 0.0 bc	1.9 ± 0.2 cd	1.5 ± 0.1 c	61.3 ± 2.5 c	Nd
	F_2	1.5 ± 0.1 b	0.3 ± 0.0 d	1.9 ± 0.1 bc	1.9 ± 0.1 cd	1.5 ± 0.1 c	72.3 ± 1.5 bc	Nd
	M_2	0.9 ± 0.1 bc	1.4 ± 0.1 b	2.0 ± 0.0 c	1.7 ± 0.0 c	1.1 ± 0.1 b	65.1 ± 0.1 c	Nd
	M_3	0.7 ± 0.2 c	1.3 ± 0.1 b	1.9 ± 0.0 bc	1.7 ± 0.1 c	1.0 ± 0.2 b	67.8 ± 0.5 c	Nd
Stress	TBR1	2.1 ± 0.0 a	0.6 ± 0.0 c	1.1 ± 0.0 a	1.0 ± 0.0 a	0.5 ± 0.0 a	21.4 ± 2.7 d	46.3 ± 0.6 a
	KD18	0.9 ± 0.1 bc	0.6 ± 0.0 c	2.3 ± 0.1 d	1.5 ± 0.0 b	1.5 ± 0.1 c	13.9 ± 0.4 d	Nd
	F_1	1.5 ± 0.0 b	0.9 ± 0.1 bc	1.8 ± 0.2 bc	1.9 ± 0.2 cd	1.3 ± 0.0 bc	25.7 ± 1.4 d	Nd
	F_2	1.5 ± 0.2 b	0.8 ± 0.2 bc	1.8 ± 0.0 bc	1.9 ± 0.1 cd	1.2 ± 0.1 bc	20.6 ± 3.0 d	Nd
	M_2	1.5 ± 0.1 b	2.5 ± 0.1 a	1.1 ± 0.1 a	1.0 ± 0.0 a	0.7 ± 0.0 ab	104.7 ± 3.1 a	34.9 ± 1.8 b
	M_3	1.0 ± 0.1 bc	1.6 ± 0.0 ab	1.5 ± 0.0 b	1.2 ± 0.1 ab	0.5 ± 0.2 a	102.5 ± 2.5 a	20.5 ± 1.5 c

Values are means ± SE (standard errors); nd: not detected. TPC: total phenol content; TFC: total flavonoid content; DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate; ABTS: 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; NO: nitric oxide; MA: momilactone A; MB: momilactone B. Mean with same letters in a column is not significantly different ($p < 0.05$).

No remarkable difference in antioxidant capacities between TBR1 and M_2 was observed (Table 2). The TPC and TFC values of the M_2, M_3, F_1, and F_2 individuals were either significantly higher or lower than that of its parent KD18 and recurrent parent TBR1 (Table 2). Subsequently, the content of MA in TBR1 was higher than KD18, but M_2 and M_3 show much greater content of MA than their parental cultivars do (104.7 and 102.5 ng/g, respectively). Interestingly, no trace of MB was found in KD18, but the salinity stress induced MB in TBR1 (46.3 ng/g), M_2 (34.9 ng/g), and M_3 (20.5 ng/g) (Table 2). Findings of Table 2 indicate that the MNU treatment also caused the uniparental inheritance of beneficial phytochemicals, including antioxidant activity and MA and MB from TBR1 to F_2 generation.

3.2. Correlation of Physiological Parameters between Progeny and Parental Lines

The distributions of examined traits of the M_2 population are shown in Figure 1. A high correlation of 0.81 ($R^2 = 0.6494$) was observed between M_2 and TBR1, while in contrary a much lower correlation of 0.45 ($R^2 = 0.2059$) was found between M_2 and KD18. The evidence indicates that agronomical and chemical properties of progenies are uniparentally inherited from the female cultivars.

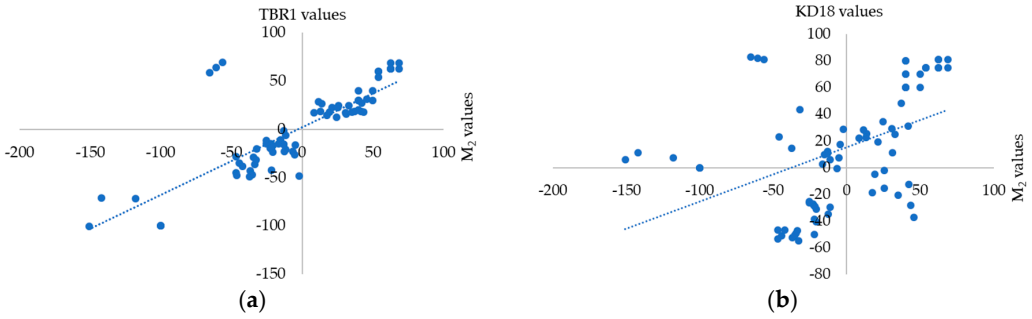


Figure 1. Distributions of phenotypic and chemical parameters between (**a**) M_2 and TBR1 ($R^2 = 0.6494$); (**b**) M_2 and KD18 ($R^2 = 0.2059$).

3.3. Genetic Segregation of F_2, M_2 and M_3 Populations

A total of 228 rice plants were used to analyze the segregations in the F_2, M_2 and M_3 generations (Figures 2 and 3). The results show that in the amplification of all the polymorphic SSR markers, the F_2 population possess both the male parent allele KD18 and the female parent allele TBR1 (following the

Mendelian theory). However, both M_2 and M_3 generations are completely inherited (100%) from the recurrent parent TBR1 cultivar (Figure 3a,b). It was observed that the preliminary treatment of MNU on the original seeds from the cross TBR1 × KD18 induced the salinity tolerance in the M_2 and M_3 genotypes, which are completely inherited from the recurrent parent TBR1 cultivar. The uniparental inheritance of salinity tolerance in the M_2 generation was completely stabilized in M_3 generation, with no segregation observed (Figure 3b).

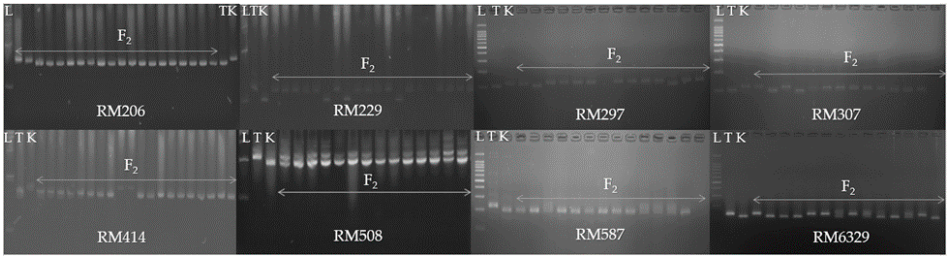
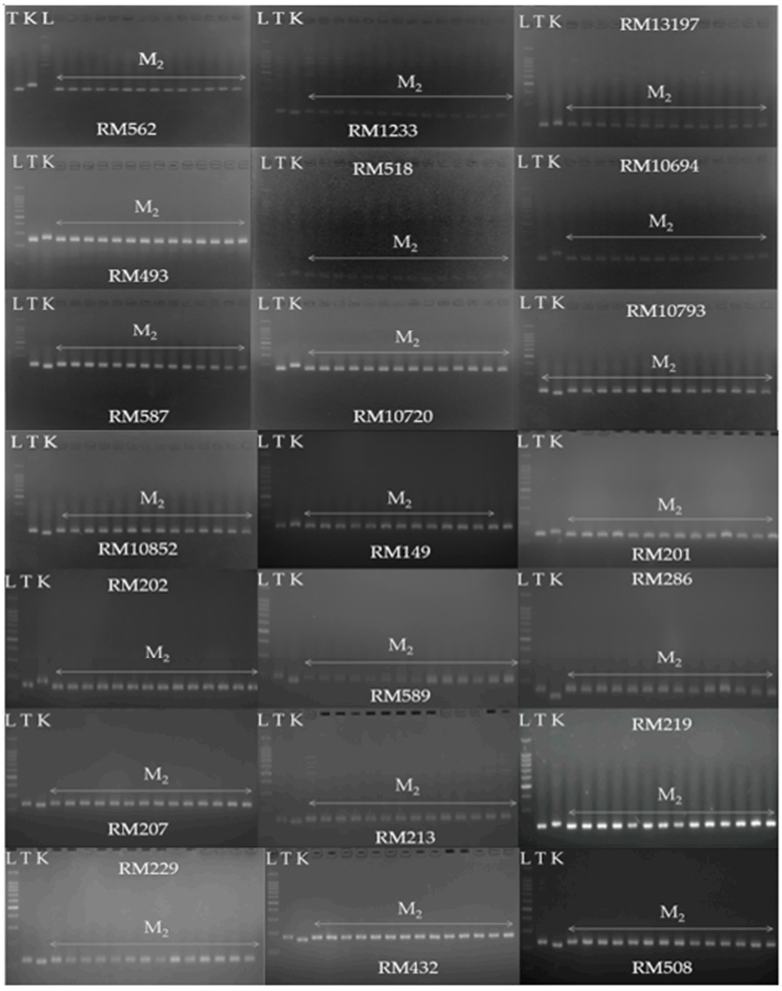
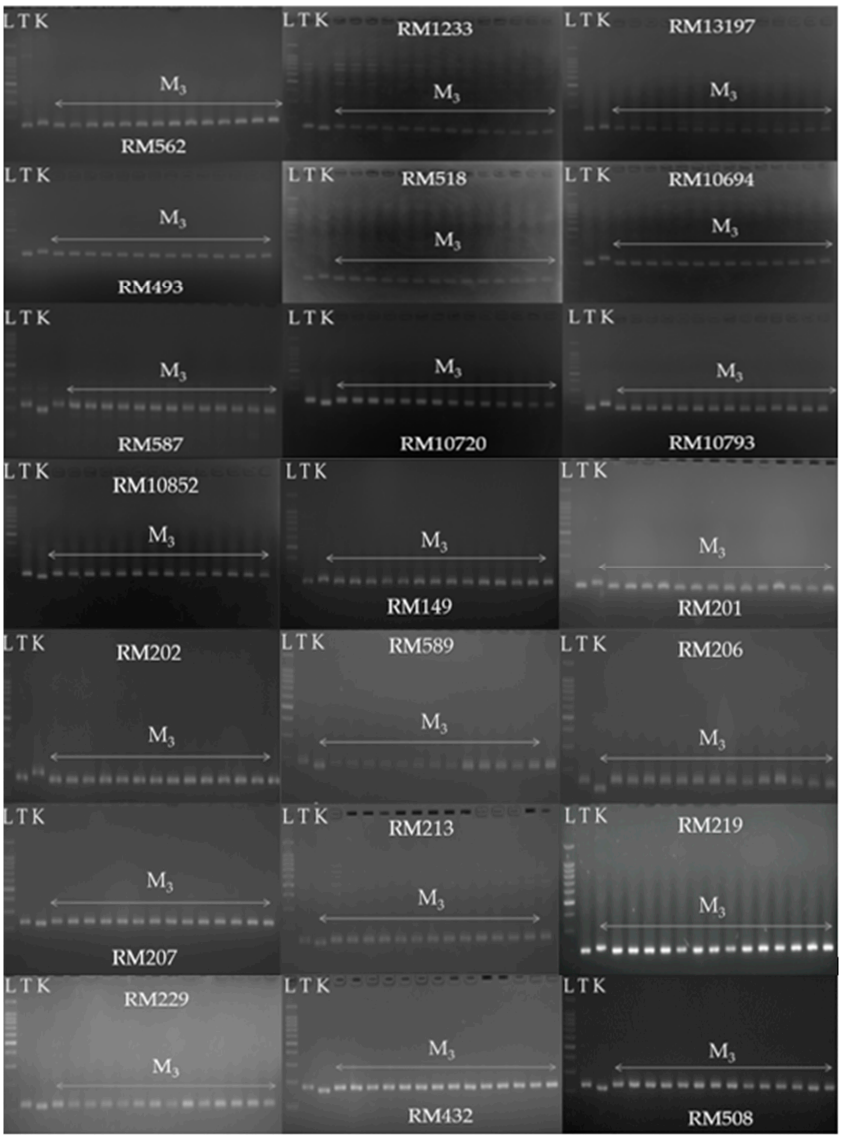


Figure 2. PCR products of F_2 generation by simple sequence repeat (SSR) markers RM206, RM229, RM297, RM307, RM414, RM508, RM587, and RM6329 (L: DNA ladder, T: TBR1, K: KD18, F_2: F_2 progenies).



(**a**)

Figure 3. *Cont.*



(**b**)

Figure 3. (**a**) PCR products of M_2 population by SSR markers RM562, RM1233, RM13197, RM493, RM518, RM10694, RM587, RM10720, RM10793, RM10852, RM149, RM201, RM202, RM589, RM206, RM207, RM213, RM219, RM229, RM432, and RM508 (L: DNA ladder, T: TBR1, K: KD18, M_2: M_2 progenies). (**b**) PCR products of M3 population by SSR markers RM562, RM1233, RM13197, RM493, RM518, RM10694, RM587, RM10720, RM10793, RM10852, RM149, RM201, RM202, RM589, RM206, RM207, RM213, RM219, RM229, RM432, and RM508 (L: DNA ladder, T: TBR1, K: KD18, M3: M3 progenies).

4. Discussion

The preliminary treatment of MNU on the original seeds from the cross TBR1 × KD18 induces the uniparental inheritance of salinity tolerant *Saltol* QTL from TBR1 to the M_2 and M_3 generations in both phenotypes (Tables 1 and 2) and genotypes (Figures 2 and 3). The genotype of the M_3 was completely similar to M_2 (Figure 3). Following the salinity tolerance, antioxidant activities and contents of MA and MB were also uniparentally inherited (Table 2), although the associated genetic markers with QTLs/genes determining antioxidant activities and biosynthesis of MA and MB were not examined in this study.

In this study, the F_1 seeds are the progenies of the cross between TBR1 (female variety) × KD18 (male variety), and the M_1 generation was induced by treating F_1 with MNU treatment. The M_1 was self-pollinated to obtain M_2, and M_3 was induced by the M_2 self-pollination. The purpose of this was to check the stability of uniparental inheritance of the salinity tolerant *Saltol* QTL from the mother variety. In conventional breeding, the salinity tolerant cultivar should be the father cultivar. Because the salinity tolerant characteristics are determined by multiple genes/QTLs, they follow the Mendel rules with complicated segregation from the F_2 generation. Therefore, to stabilize the salinity tolerance, F_2 seeds are commonly crossed with the father variety (backcross) and repeated in many generations which requires huge amounts of fieldwork, time, and money [11,12,48–52]. All previous work used the donor (male) cultivars having the *Saltol* QTL in the breeding to introduce the salinity tolerance to the target cultivars. For instance, Babu et al. [11] developed a rice line Pusa1734-8-3-3 having good salinity tolerance in the seedling stage compared with the FL478 donor (father cultivar). The salinity tolerance of Pusa1734-8-3-3 was the $BC_3F_4{}^+$ (>7 generations) from the cross Pusa Basmati 1121 (female cultivar) and FL478 (male cultivar). Similarly, Leon et al. in 2017 developed different salinity tolerant introgression lines (Ils) from the BC_4F_4 (eight generations), but the salinity tolerance characteristic has not yet been stabilized [48]. The treatment of MNU in this study induced the uniparental inheritance for the *Saltol* QTL, providing a simple protocol to develop rice lines tolerant to salinity.

To date, almost all known QTLs involved in salt-tolerance are located on chromosome 1 [7]. In this study, the selected nine SSR markers relevant to salinity tolerant are polymorphic and stationed on chromosomes 1, 2, 3, 4, 5, 7, 8, 10 and 11, in which the major SSR markers are located on the chromosome 1 (Supplementary Table S2). In this study, we did not carry out a vast screening on many SSR markers but selected only fifty known SSR markers related to important agronomic, pest resistant and salinity tolerant *Saltol* QTL, which have been already reported in previous research (Supplementary Table S2). These markers were used to distinguish the difference in parental genotypes associated with the phenotypes observed in Table 1. Subsequently, twenty-one SSR markers were found to be polymorphic and involved in salinity tolerant and elite agronomic traits (amylose content, plant height, spikelet fertility, brown plant hopper resistance, blast disease resistance) (Supplementary Table S2). Therefore, they were used to examine the F_2, M_2, and M_3 individuals. However, only nine SSR markers involved in *Saltol* QTL are polymorphic, including seven SSR markers locate on chromosome 1 (RM493, RM562, RM10694, RM10720, RM10793, RM10852, and RM13197), and two markers RM201 and RM149 located on chromosome 8 and 9, respectively [8–10] (Figure 2; Supplementary Table S2). Other polymorphic markers were identified relating to plant height (RM202, RM206, RM219, RM229) [12], leaf diameter (RM508) [43], spikelet fertility (RM202, RM206) [12] spikelet number (RM229) [12], grain yield (RM219, RM432) [12], amylose content (RM219, RM432, RM508, RM587, RM589) [12], gel consistency (RM589) [12], blast resistance (RM1233, RM206, RM207, RM213) [12,43], brown planthopper resistance (RM206) [12], and water and nitrogen use efficiency (RM518) [45] (Supplementary Table S2). Other SSRs relevant to the *Saltol* QTL are not polymorphic and they could not be used to identify uniparentally controlled by MNU treatment (Supplementary Figure S3). Further treatments of MNU should be applied to enable the uniparental inheritance of more salinity tolerant genes/QTLs, such as *OsCPK*17, *OsRMC*, *OsNHX*1, *OsHKT*1;5 genes [6], except the *Sal*T gene derived from the *Saltol* QTL.

It is proposed that only these nine SSR markers relevant to the *Saltol* QTL are responsible for the salinity tolerance of the TBR1 × KD18 cross, and momilactones MA and MB are determined by five known biosynthesis genes (*AK103462*: dehydrogenase gene and *CYP99A2* and *CYP99A3*: P-450 genes form a chitin oligosaccharide elicitor, together with *OsKS4* and *OsCyc1*, the diterpene cyclase genes) clustered in chromosomes 2 and 4 [53]. Following the Mendelian genetic rules, because all these genes are located on chromosomes in the rice nucleus, the segregation ratios should be theoretically 1/1024 (5 genes) × 1/262,444 (9 genes) = 1/268,435,456 recombinants. Therefore, this study helps to reduce the huge laborious work, cost, and long breeding time that conventional breeding requires [12,25].

In our previous study, a total of 28 polymorphic SSR markers were selected from 200 markers relevant to plant height, semi-dwarfism, amylose content, protein content, gel consistency, grain yield,

and spikelet fertility were genotyped on the second generation of similar cross TBR1 (mother) × KD18 (father) [12]. These markers are distributed on chromosomes 1–7, 9, and 11. All phenotypes and genotypes of the abovementioned growth and quality characteristics were uniparentally inherited in the second generation [12]. In addition, PCR results showed identical results of this study, indicating that all the 28 SSR markers were completely inherited from the recurrent TBR1 cultivar and no segregation was observed [12]. Combining the results obtained from this research, it can be concluded that by the prior treatment of MNU on F_1 seeds, important growth, quality traits, and the salinity tolerant *Saltol* QTL of the TBR1 cultivar can be uniparentally inherited to the F_2 and F_3 generations. Study on the ability of this promising rice source on other abiotic stresses is suggested. However, the subsequent generations should be strictly examined to ensure the stability of the uniparental inheritance. The mechanism of inducing this novel uniparental inheritance for salinity tolerance by MNU application needs to be further analyzed.

This study also shows that in salinity stress, rice plants promote the levels of antioxidant activities as well as content of MA and MB (Table 2). In a non-treated control, no trace of MB was observed, but in the salinity treated condition, TBR1, M_2, and M_3 induced MB as well as promoted MA content (Table 2). Although it is observed that phenotypically, antioxidant activities and induction of MA and MB in rice plants are also uniparentally inherited, polymorphic SSR markers related to antioxidant potentials and biosynthesis of MA and MB should be screened to evaluate whether their genotypes can be also uniparentally inherited or not. However, this study highlights that the preliminary treatment of MNU can also induce levels of antioxidant activities and important phytochemicals including MA and MB in rice, and the induction of antioxidant activities and MA and MB is also uniparentally inhibited to the second generation (Table 2).

MA and MB were first isolated and identified by Kato et al. [54] who reported that these two compounds are the growth inhibitors (allelochemicals) and phytoalexins [55,56] in rice. For more than 40 years since 1973, scientists worldwide have acknowledged that MA and MB are allelochemicals and conducted various experiments on examining quantities of MA and MB in different rice cultivars, as well as in rice organs and the release from roots [57,58]. However, the application of allelochemicals including MA and MB is questionable as the released amounts from the root leaches and plant parts are too low to inhibit growth of weeds, although they showed promising reduction on weed growth in laboratory experiments [28]. For MA and MB, we recently found that the two compounds are involved in drought and salinity tolerance in rice rather than allelochemicals [28,59]. In addition, MA and MB have the potential to control diabetes [60], cancers [61–63], and skin diseases [30]. In this study, MB was not found in the control condition, however the salinity stress induced MB in the recurrent parent (TBR1), M_2, M_3, and MA in all tested samples (Table 2). Therefore, it is concluded that treatment of MNU induced uniparental inheritance for both the salinity tolerant *Saltol* QTL, as well as beneficial phytochemicals, including antioxidants and MA and MB, in rice.

5. Conclusions

Findings of this study reveal that the principal salinity tolerant *Saltol* QTL trait in rice can be uniparentally controlled, along with beneficial phytochemical antioxidants and MA and MB in rice. The treatment of MNU aids to speed up and simplify the breeding of new rice cultivars, having both elite agronomic traits and tolerance to environment stresses. The question of whether QTLs/genes other than the *Saltol* QTL such as *OsCPK*17, *OsRMC*, *OsNHX*1, *OsHKT*1; 5 genes can also be uniparentally inherited by MNU treatment should be further examined.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/7/1032/s1, Table S1: Comparison of color between FL478 and Pokkali; Table S2: SSR markers used for distinguishing female parent (TBR1) and male parent (KD18); Figure S1: PCR products of parents and progenies with polymorphic SSR markers. Table S2: Polymorphic SSRs for screening progeny generations; Figure S2: PCR products of progenies with polymorphic SSR markers; Figure S3: Screening of parental DNAs with involved SSR markers.

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