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Developing Green Super Rice Varieties with High Nutrient Use Efficiency by Phenotypic Selection Under Varied Nutrient Conditions

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Abstract: To develop green super rice varieties with high and stable yields under the rainfed conditions and improved nutrient use efficiency (NuUE), a modified backcross (BC) breeding approach was adopted using a high yielding and widely adaptable *Xian* variety, WTR1, as the recipient and a *Geng* variety, HAN, as the donor. Starting from the BC_1F_2 generation, the BC population had gone through one generation of selection under the IG, LI and RF conditions, followed by consecutive four generations of screening and selection for high GY under six different nutrient conditions, leading to the development of 230 BC_1F_6 introgression lines (ILs). The final evaluation of the 230 ILs under the six nutrient conditions identified many ILs with improved yields under various combinations of nutrient deficient conditions, including 12 promising lines that had significantly improved NuUE under two or more nutrient deficiency conditions. Our results demonstrated an efficient inter-subspecific BC breeding procedure with first round selection under the rainfed-drought condition followed by four generations of progeny testing for yield performances under six different nutrient conditions. The promising ILs were studied under replicated yield trials under 75N and -NPK conditions for developing high yield rice varieties with improved NuUE. Our results indicated that NuUE in rice was controlled by complex genetic and physiological mechanisms and the developed ILs provided useful materials for genetic and molecular dissection of NuUE in rice.

Keywords: nutrient use efficiency, grain yield, nitrogen, phosphorus, potassium, green super rice, BC breeding.

1. Introduction

Rice remains the most important cereal crop and principal staple food in developing countries, particularly in Asia [1]. The global rice production has to increase by about 25% by 2025 to keep the base year 2001, with a target of 732.5MT of rice produced each year. The world rice production during the Green Revolution (GR) and post-GR was more than doubled [2]. This increased rice production was primarily achieved through the development and wide adoption of rice varieties that were highly responsive to high inputs (chemical fertilizers, pesticides, and ample irrigation water) [3]. These breeding objectives predominated over the last three to four decades but now lie exhausted amidst our hope to raise productivity per se sustainably. However, the crop's efficiency in harnessing applied inputs led to yields approaching a theoretical limit [4, 5]. Single-year fertilizer N recovery efficiencies in researcher-managed experimental plots averaged 46% for rice crop [6]. However, the experimental plots do not accurately reflect the recovery efficiencies available on-farm. Scale in farming operations differences and management practices such as tillage, seeding, weed and pest control, irrigation, and harvesting usually result in lower nutrient use efficiency. Nitrogen recovery in crops grown by farmers rarely exceeds 50% and is often much lower. The average nitrogen recovery efficiency in farmer's fields ranges from 20% to 30% under the rainfed conditions and from 30% to 40% under the irrigated conditions [7]. In most of the time, only half of the applied nutrients used by rice plants and a significant part of nitrogen in the fields lost through volatilization, leaching, de-nitrification, and soil erosion [8,9].

Over the years, rice varieties have not been intentionally improved to maximize nutrient absorption. It is, therefore, necessary to breed rice cultivars with improved nutrient use efficiency (NuUE). Recently, green super rice (GSR), defined as rice varieties that can produce high and stable yields under fewer inputs (water, nutrients, and pesticides) and adverse conditions, was a new concept for enhancing rice NuUE and achieving sustainable rice production through breeding [3, 10]. Breeding varieties with higher NuUE are essential not only to improve yield and reduce production costs but also to avoid environmental pollution and maintain the sustainability of cropping systems [11]. In the light of high energy costs and increasingly fluctuating resources, future agricultural systems need to be more productive and efficient, including for fertilizer and water. Also, improved NuUE is an essential prerequisite for the extending the crop production into marginal soils with lower nutrient availability such as nitrogen, phosphorus, and potassium [11]. Thus, developing rice varieties with high grain yield under low-nutrient conditions has become a breeding priority [12]. Cultivars with higher NuUE, coupled with best management practices, will contribute to sustainable agricultural systems protecting and promoting soil, water, and air quality [13]. Unfortunately, only a few systematic breeding efforts until now have been intended in this respect. In these cases, breeding populations were screened under varying rates of N and P fertilizers for identification of NuUE varieties [14-18].

Here, we report an effort for developing GSR varieties with improved NuUE using a unique breeding approach through selecting introgression lines (ILs) with higher NuUE in a BC breeding program, which is potentially going to be widely adopted for improving NuUE of rice varieties in future.

2. Materials and Methods

2.1. Plant materials

A BC_1F_2 population developed from a cross between an elite *Xian* (indica) variety, Weed Tolerant Rice1 (WTR1, as the recipient) and a Chinese *Geng* (japonica), Hao-An-Nong (HAN as the donor) [19]. WTR1 is a high yielding from south China with wide adaptability across subtropical and tropical areas of Asia. WTR1 was crossed with HAN in the 2010 dry season (DS) and the F_1 plants

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were backcrossed to WTR1 once in the 2010 wet season (WS). In the 2011 DS, seeds from more than 25 segregating BC_1F_1 plants were bulk harvested without selection to form a single BC_1F_2 population.

2.2. Selection schemes for improving NuUE

Figure 1 shows the phenotypic selection schemes of the BC_1F_2 bulk population to develop ILs with improved NuUE at the International Rice Research Institute (IRRI at 14°13′ N and 121°15′ E, at an elevation of 21 m above mean sea level), Philippines) during the three wet seasons of 2011-2013 and two dry seasons of 2012 and 2013, respectively. The first round of single-plant selection for higher grain yield was practiced on the BC_1F_2 bulk population grown under irrigated conditions (IG), low-input (LI) and rainfed (RF) situations in 2011WS, resulting in 46 selected BC_1F_2 plants. Then, in the following seasons, four rounds of line-based selection were carried out from progeny testing under six different nutrient input conditions, including the NPK, 75N, -N, -P, -NP, and -NPK conditions. The NPK condition was the condition with total applied NPK fertilizers of 160-50-50 kg/ha^{-1} in the DS and 90-30-30 kg/ha^{-1} in the WS. The 75N, -N, -P, -NP, and -NPK conditions indicated that 75% of nitrogen, the absence of N, the absence of P, the absence of both N and P, and finally absence of NPK were used, respectively, compared with the normal NPK condition. In each season, seeds of each selected line and parents were sown on the seedling nursery, and 24 20-day old seedlings of each line were transplanted into a two-row plot at a spacing of 20.0 cm × 15.0 cm with one seedling per hill. The selection was practiced by selecting the best 1-3 plants from the best yield performing lines.

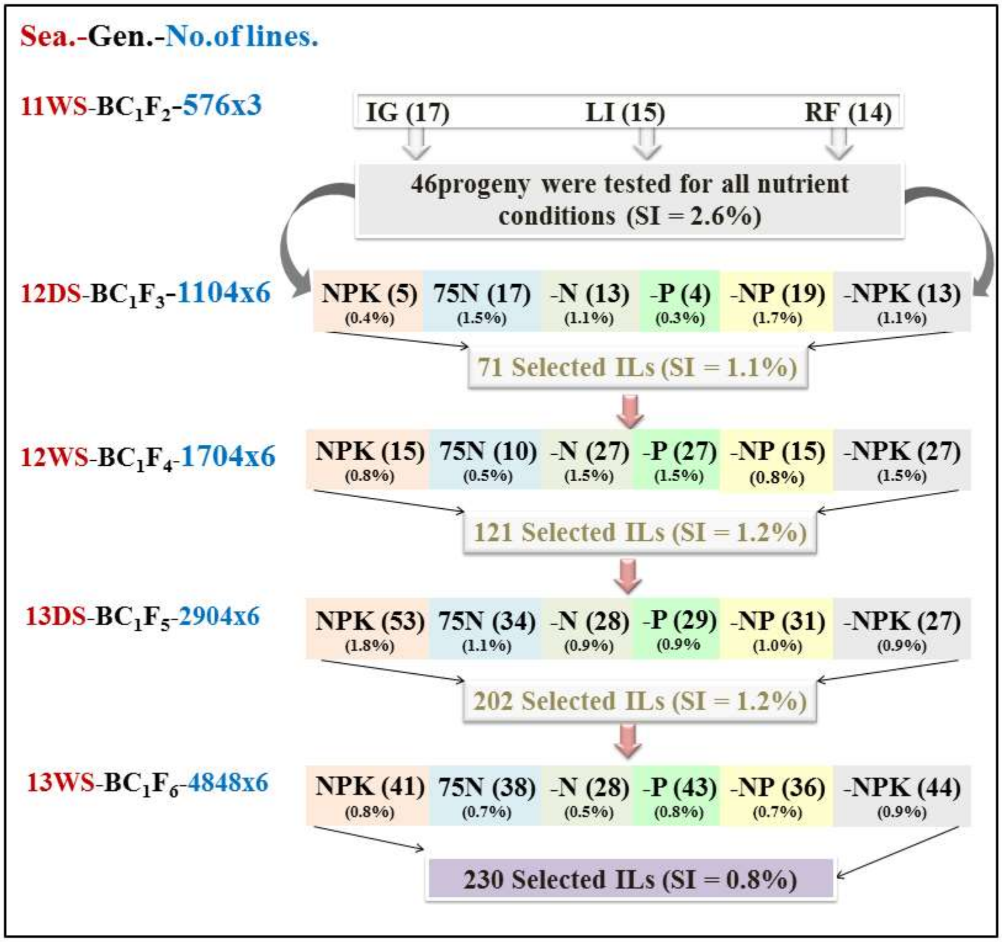

Figure 1. Phenotypic selection schemes of the BC1F2 bulk populations to develop 230 nutrient use efficient ILs [**WTR1** = Weed Tolerant Rice 1 (recipient); **HAN** = Hao-an-nong (the donor); **IG** = the

irrigated conditions; **LI** = the low-input conditions; **RF** = the rainfed conditions; **ILs** = introgression lines; **SI** = selection intensity; **Sea** = season; **Gen** = generation; **No. of lines** =number of lines and grown under six nutrient conditions; **DS** = dry season and **WS** = wet season]

Phenotyping of grain yield attributed traits of NuUE-ILs: The final evaluation of the 230 BC_1F_7 ILs for NuUE was conducted in replicated field experiments under the same six levels of the nutrient input: NPK, 75N, -N, -P, -NP, and -NPK conditions at IRRI in the 2013DS. Seeds of the 230 ILs and parents were sown on the seedling nursery, and 20-day old seedlings of each line were transplanted into a two-row plot with 24 plants or hills at a spacing of 20.0 cm × 15.0 cm, with one seedling per hill and two replications for each line. Weeds were controlled by using herbicides and hand pulling methods. At the maturity, three plants were diagonally sampled in the middle row of each plot for the phenotypic evaluation of 13 agro-morphological and grain yield traits. These traits included plant height (PH, cm), flag leaf length (FLL, cm), flag leaf width (FLW, mm), flag leaf area (FLA, cm2), tiller number (TN), heading date (HD), spikelet number per panicle (SN), panicle length (PL, cm), filled grain number per panicle (FGN), spikelet fertility (SF), 1000-grain weight (TGW), biomass (BM, g), and grain yield per plant (GY, g).

Statistical data analysis: Data of the measured morphological, agronomical and yield traits were analyzed using Microsoft Excel software. Analysis of variance (ANOVA) was used to determine the significant differences among different treatments (T) of (NPK, 75N, -N, -P, -NP, and -NPK) nutrient input, among lines (G), and GxT interactions using SAS program 9.1 (SAS Institute Inc, Cary Nc). Duncan Multiple Range Tests (DMRT) was used to compare the differences between the mean values of the treatments and genotypes with the help of R software [20].

3. Results

3.1. Development of introgression lines (ILs)

Fig. 1 shows the selection scheme for developing WTR1 ILs starting with the first round of selection of the WTR1/HAN//WTR1 BC_1F_2 population with 576 plants under each of the IG, LI and RF conditions. In the first round selection, 46 plants were visually selected based on the desirable plant type and better yield performances than WTR1 (selection intensity, SI = 2.7%) in 2011WS, including 17 plants from IG, 15 plants from LI, and 14 plants from RF. In the next season of 2012DS, the 46 BC1F3 lines (1,104 plants) were planted in single lines under the six different nutrient conditions, from which 71 plants were selected, including 7, 17, 13, 4, 19 and 13 plants from the NPK, -N, -P, -NP and -NPK conditions, respectively. In the following three consecutive seasons of 2012WS, 2013DS, and 2013WS, the same phenotypic selection was performed, resulting in 121 BC_1F_4 plants, 202 BC_1F_5 plants, and 230 BC_1F_6 plants, respectively. Taking together, the cumulated contribution of the four rounds of selection through progeny testing under the NPK, -N, -P, -NP and -NPK conditions leading to the final 230 BC1F7 (ILs) was 0.107 for NPK, 0.144 for 75N, 0.180 for -N, 0.088 for -P, 0.208 for -NP and 0.273 for -NPK.

3.2. Phenotypic performances of ILs for agro-morphological and yield traits in diverse environments

In the replicated progeny testing, the recipient and donor had similar average grain yield across all six nutrient input conditions except under -NPK and -P. In the former case, WTR1 had significantly higher GY than the donor, HAN, while the opposite was true in the latter case under-P (Table 1). When the effects of different nutrient treatments were compared, the mean yields of the nutrient treatments could be divided into three levels: (1) the normal NPK showing the highest mean yield of the tested materials, and showed non-significant differences to 75N; (2) -P having the second highest average yield; and (3) -N, -NP, and -NPK showing the similarly low average GY for the tested lines. The nutrient treatments had similar effects on PH, TN, and biomass and had no effects on HD, TGW and SF (Fig. 2). However, the deficiency of nitrogen (-N) and phosphorus (-NP) caused significant

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reductions in SN, FNP, FLL, and FLA in comparison to the remaining five NuUE conditions. Importantly, the ILs showed similar mean values but tremendous variation and transgressive segregation for GY under all six nutrient conditions.

Table 1. The mean grain yield performances of 230 ILs and their parents under six nutrient input conditions

NuUE conditions	WTR1	HAN	Difference	ILs		DMRT comparison
				Range	Mean	
-N	21.0	21.6	-0.6	13.9~35.0	21.6±3.8	A
-NP	20.5	18.3	2.2	11.9~40.5	20.5±4.5	A
-NPK	21.4	17.9	3.5*	12.8~43.0	20.5±4.5	A
-P	25.9	28.4	-2.5*	17.6~42.2	26.8±4.8	B
75N	29.1	30.1	-1.0	20.8~48.8	32.6±5.5	C
NPK	32.8	31.9	0.9	19.3~50.3	32.5±5.2	C
Mean	25.1±5.1	24.7±6.2	0.4			

DMRT=Duncan's multiple range tests for comparison of treatments; different letters and * indicate significant differences at $P \leq 0.05$, while the same letter indicates non-significant differences.

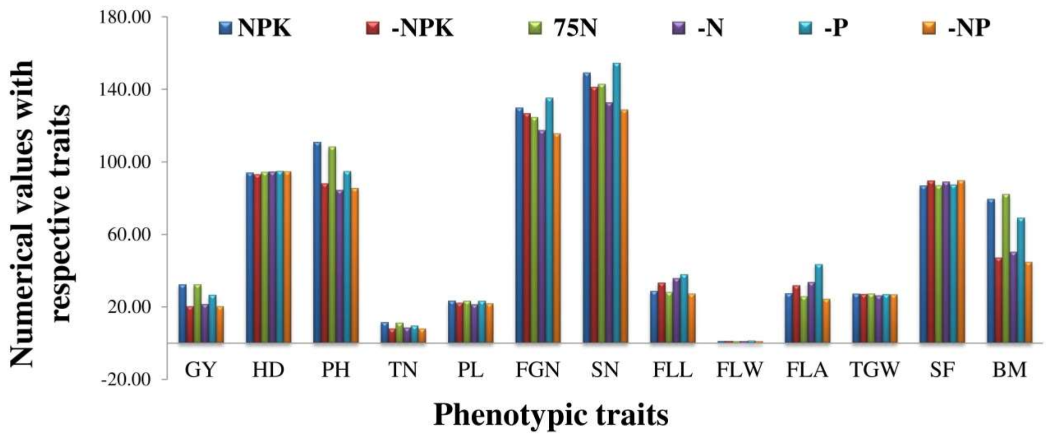

Figure 2. Effects of six nutrient conditions on the average expressions of grain yield per plant (in g, GY) and related agro-morphological and yield attributed traits of 230 WTR1 introgression lines

GY=grain yield per plant(g); **HD**=heading date(days); **SN**=spikelet number per panicle; **GN**=filled grain number per panicle; **SF**=spikelet fertility(%); **TGW**=thousand-grain weight(g); **PH**=plant height(cm); **TN**=tiller number per plant; **PL**=panicle length(cm); **FLL**=flag-leaf length(cm); **FLW**=flag-leaf width(cm); **FLA**=flag-leaf area(cm^2); **BM** biomass(g)

Table 2 shows the correlation coefficients between the mean yield performances and other agronomic traits measured under different nutrient conditions. High and positive correlations between grain yield and biomass were observed across all six nutrient conditions, particularly under the -N, -NP and -NPK, which was followed by FLW, FLA, and TN. Surprisingly, contributions of the two other direct yield components (FGN and TGW) to grain yield were, though positive, much less important, particularly under the less nutrient deficiency conditions (NPK and 75N).

Table 2 Correlations of GY with other related traits of 230 WTR1 introgression lines under six nutrient conditions

GY	HD	SN	FGN	SF	TGW	PH	TN	PL	FLL	FLW	FLA	BM
-N	-0.12	0.03	0.10	0.18**	0.17*	0.20**	0.20**	0.23***	0.09	0.19**	0.18**	0.35***
-NP	-0.16*	0.31***	0.31***	0.02	0.09	0.52***	0.62***	0.39***	0.33***	0.52***	0.50***	0.74***
-NPK	-0.17*	0.21**	0.27***	0.14*	0.24***	0.40***	0.60***	0.39***	0.41***	0.56***	0.57***	0.73***
-P	-0.04	0.19**	0.20**	0.11	0.36***	0.12	0.25***	0.24***	-0.01	0.11	0.06	0.77***
75N	-0.12	0.14*	0.15*	0.08	0.09	0.08	0.18**	0.24***	0.09	0.07	0.11	0.26***
NPK	-0.09	0.18**	0.22***	0.14*	-0.01	-0.05	0.16*	0.13*	-0.08	-0.17**	-0.16*	0.59***

*, **, *** indicate significant level at 0.05, 0.01, and 0.001 respectively.

The ILs showed tremendous segregation for their GY under different nutrient treatments (Table 2). In fact, a significant portion of ILs had significantly (10%) higher GY than WTR1 under each of the nutrient treatments. In particular, more ILs showed significantly improved yields under the 75N and -NP conditions, while the opposite was true under -P. Under the other conditions, the ILs showed largely normal distributions with approximately equal numbers of high and low yield ILs, providing tremendous opportunities for selection. Indeed, several promising ILs with significantly higher GY than WTR1 were identified under each of the NuUE treatments. These included Nue-114 (50.6 g), Nue-115 (42.9 g), Nue-3 (42.1 g), Nue-51 (42.1 g), Nue-112 (40.4 g) and Nue-230 (48.8 g) under the NPK, -NPK, -N, -P, -NP, and 75N conditions.

3.3. Selection of promising ILs with superior yields under two or more nutrient conditions

Table 3 shows the numbers of the selected 230 ILs from previous screening under the IG, LI and RF conditions that had significantly higher mean grain yields under one or two nutrient conditions. Of the 230 selected ILs, the number of ILs showing significantly higher GY than WTR1 was 52 under NPK, 103 under 75N, 33 under -P, 63 under -N, 52 under -NP, and 49 under -NPK, respectively. This translated into indirect selection efficiencies of 0.226, 0.448, 0.143, 0.274, 0.226 and 0.213 for improved yield performances under the NPK, 75N, -P, -N, -NP and -NPK conditions, respectively. Of the 52 high yielding ILs identified under the normal NPK conditions, 26, 7, 17, 20 and 17 ILs also showed significantly improved GY under the 75N, -P, -N, -NP, and -NPK conditions, respectively. Of the 103 high yielding ILs selected under 75N, 20, 31, 34, 31 and 26 ILs showed significantly improved GY under the normal NPK, -P, -N, -NP, and -NPK conditions, respectively. Of the 33 high yielding, ILs identified under the -P condition, 20, 7, 13, 15 and 9 of these ILs performed well with significantly improved GY under the normal NPK, 75N, -N, -NP, and -NPK conditions, respectively. Similarly, of the 63 high yielding ILs selected under the -N condition, 13, 31, 17, 22 and 20 of these ILs showed significantly improved GY under the -P, normal NPK, 75N, -NP, and -NPK conditions, respectively. From the 52 high yielding ILs selected under the -NP condition, 22, 15, 34, 20, we were able to identify 20 ILs showing significantly improved GY under the -N, -P, normal NPK, 75N, and -NPK conditions, respectively. Finally, from the 49 high yielding ILs under -NPK, 20, 27, 9, 31 and 17 ILs also showed significantly improved yields under the NPK, 75N, -P, -N, and -NP conditions, respectively. This could be converted into an average secondary indirect selection efficiency of 0.335 for NPK, 0.276 for 75N, 0.388 for -P, 0.327 for -N, 0.454 for -NP and 0.424 for -NPK, respectively. In other words, replicated progeny testing under the -NP and -NPK had resulted in the highest efficiency for selecting improved grain yields across all six nutrient conditions.

Table 3 Performances of WTR1 introgression lines (ILs) with 10% (statistically significantly) higher and lower GY than the recipient

NuUE	10% higher	10% lower

conditions	No.	Mean±SD	Range	No.	Mean±SD	Range
NPK	52	39.9±3.3	36.1~50.3	51	26.2±2.3	19.3~28.7
75N	103	37.7±3.2	33.2~48.8	23	23.9±1.6	20.8~25.7
-P	33	35.2±3.2	31.4~42.2	58	21.3±1.6	17.6~23.3
-N	63	26.5±2.7	23.8~35.0	57	17.4±1.2	13.9~18.9
-NP	52	26.8±4.3	22.6~40.5	28	14.7±1.2	11.9~16.4
-NPK	49	27.2±3.8	23.6~43.0	37	15.0±0.9	12.8~16.1

Table 4 shows the numbers of low yielding ILs under different conditions from all high yielding ILs identified under each of the six nutrient conditions. Generally, a small portion of the ILs showing superior yields under one nutrient condition was yielding poorly under the other condition(s), suggested a low correlation for grain yield performances between different nutrient conditions.

Table 4 The numbers of WTR1 introgression lines showing significantly higher grain yields under two nutrient conditions

NuUE conditions	NPK	75N	-P	-N	-NP	-NPK
NPK	52	26	7	17	20	17
75N		103	20	31	34	31
-P			33	13	15	9
-N				63	22	20
-NP					52	27
-NPK						49

The values on the first diagonal show the numbers of higher GY ILs in each of the nutrient condition and values in the upper triangular show the numbers of higher GY ILs shared in each pair of the nutrient conditions.

3.4. GY performances of groups divided by the type of first-round selection

To examine how the types of the first round selection affected the selection efficiency for improving NuUE, the 230 ILs were divided into three groups, including 43 ILs originally selected from the IG conditions, 21 ILs from the LI conditions, and 166 ILs from the RF conditions. Surprisingly, ILs originally selected from the LI conditions showed the lowest mean GY under -NP, -NPK and -P, but highest mean GY under 75N and NPK, indicating that these ILs adapted better under good nutrient conditions, but very sensitive to P deficiency (Fig. 3). In contrast, those ILs originally selected from the IG conditions showed relatively better GY under -N and -NPK but performed poorly under 75N and NPK, suggesting they were less sensitive to N deficiency, while those ILs originally selected from the RF conditions appeared to perform slightly better under the -P conditions.

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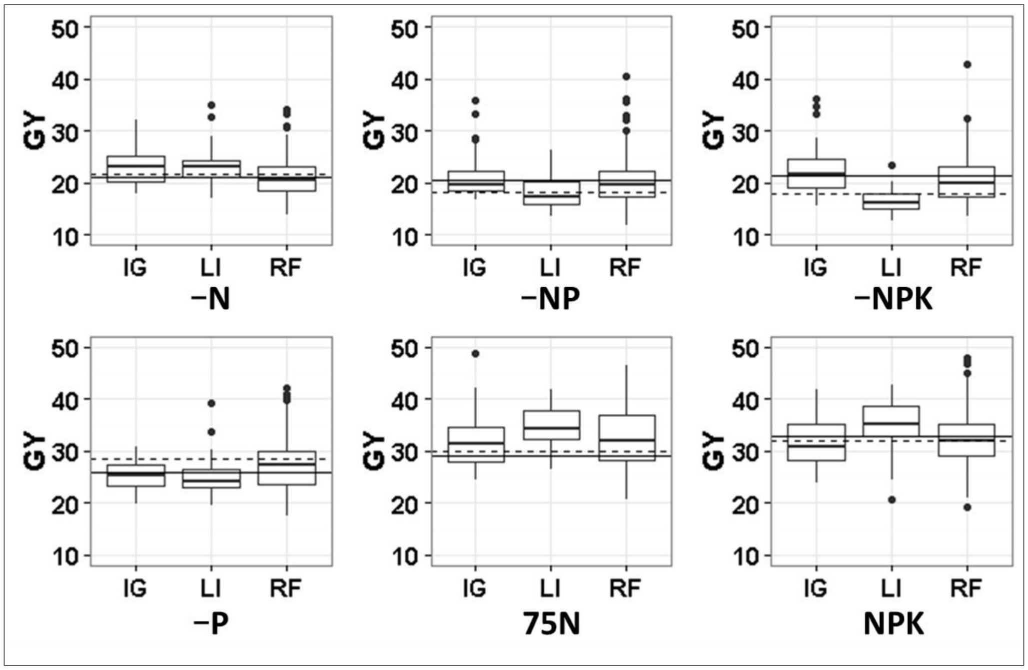

Figure 3. Comparison of mean GY performances between introgression line groups divided by the type of first-round selection, in which solid and broken lines indicate the values of the recipient, WTR1, and the donor, HAN, respectively. [**IG** = irrigated; **LI** = low input; **RF** = rainfed (drought)]

We indeed observed a negative correlation between yield performances under -P and -N conditions in some ILs. For example, five high yielding ILs (Nue-104, Nue-46, Nue-57, Nue-77, and Nue-83) identified under the -P conditions showed poor yield performances under the -N conditions (Table 6). When compared with WTR1, the poor yield performances under -N could be primarily attributed to their greatly reduced biomass, TN, and FGN.

Table 6. Grain yield performances of five WTR1 ILs and their parents with high GY in the -P condition but low GY in the -N condition

Entry	NuUE Con.	GY	HD	PH	TN	SN	FGN	SF	TGW	PL	FLL	FLW	FLA	BM
WTR1	-P	25.9	91.5	96.7	13.2	199.6	158.3	87.6	24.9	24.2	41.2	1.5	46.8	94.7
HAN		28.4	90.5	89.0	10.0	149.4	137.1	91.2	22.6	23.4	37.2	1.5	41.4	69.9
WTR1	-N	21.0	96.0	100.4	12.5	161.6	130.0	78.1	25.7	19.9	39.2	1.2	36.3	75.1
HAN		21.6	87.0	81.6	13.5	112.4	106.9	96.6	27.1	21.7	38.2	1.3	37.7	128.7
Nue-104	-P	31.7	96.0	96.7	11.5	152.3	129.0	85.0	26.9	23.9	38.4	1.6	44.7	71.4
Nue-46		31.7	93.5	93.3	8.7	142.9	127.7	89.4	30.3	22.9	37.2	1.7	46.1	57.0
Nue-57		33.6	95.5	97.0	8.3	153.5	131.4	85.2	33.0	23.9	33.7	1.6	40.4	80.8
Nue-77		39.9	96.0	96.5	14.0	150.4	132.0	87.8	31.0	24.3	38.4	1.5	42.8	83.3
Nue-83		35.3	95.0	92.3	10.5	173.1	156.7	90.4	28.8	24.0	38.4	1.7	48.1	110.2
Mean		34.4	95.2	95.2	10.6	154.4	135.4	87.5	30.0	23.8	37.2	1.6	44.4	80.5
Nue-104	-N	17.0	94.5	84.3	9.2	149.9	138.5	94.2	26.5	22.6	35.9	1.3	35.9	54.7
Nue-46		16.8	93.5	86.4	9.7	122.3	107.9	92.7	28.1	22.4	34.6	1.2	32.0	50.5

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Nue-57	18.1	93.5	79.4	12.2	126.6	92.4	81.3	28.7	21.8	36.8	1.3	36.4	39.9
Nue-77	18.5	94.0	83.8	8.3	121.5	109.3	89.8	29.4	21.4	37.4	1.3	37.0	49.1
Nue-83	16.6	96.5	82.6	8.3	102.5	88.6	89.8	25.1	20.2	33.0	1.2	29.3	40.3
Mean	17.4	94.4	83.3	9.5	124.6	107.4	89.6	27.5	21.7	35.6	1.3	34.1	46.9

Con=conditions; **GY=**grain yield per plant(g); **HD=**heading date(days); **SN=**spikelet number per panicle; F**GN=**filled grain number per panicle; **SF=**spikelet fertility(%); **TGW=**thousand-grain weight(g); **PH=**plant height(cm); **TN=**tiller number per plant; **PL=**panicle length(cm); **FLL=**flag-leaf length(cm); **FLW=**flag-leaf width(cm); **FLA=**flag-leaf area(cm^2); **BM=**biomass(g)

3.5. Promising ILs under different NuUE conditions

Table 7 lists 12 ILs that showed significantly higher grain yields than WTR1 under two or more nutrient conditions and the same yields as WTR1 under the other conditions. These included one promising line, Nue-115, which had significantly improved yields under all four nutrient deficient conditions, four ILs (Nue-114, Nue-112, Nue-229, and Nue-230), that had superior yield performances under the normal or N75 plus two nutrient deficiency conditions. These lines have now promoted to the replicated multi-location yield trials for further testing for potential releases as new varieties in the target environments of several Asian and African countries.

Table 7 Twelve promising higher ILs with superior yield performances in two or more nutrient conditions

S. No	ILs	Designation	NuUE Combinations					
			NPK	75N	-N	-NP	-NPK	-P
1	Nue-57	GSR IR2-1-L1-NU1-NU1-NU1-NU1	√	√	-	-	-	-
2	Nue-60	GSR IR2-1-RF6-NU3-NU4-NU68-NU35	-	√	-	-	-	√
3	Nue-77	GSR IR2-1-RF6-NU3-NU4-NU7-NU38	-	-	-	√	-	√
4	Nue-86	GSR IR2-1-RF6-NU4-NU7-NU60-NU56	-	-	-	√	-	√
5	Nue-106	GSR IR2-1-RF6-NU4-NU9-NU14-NU66	√	√	-	-	-	-
6	Nue-118	GSR IR2-1-RF6-NU6-NU1-NU20-NU88	√	-	√	-	-	-
7	Nue-228	GSR IR2-1-RF6-NU7-NU2-NU77-NU94	-	-	-	√	√	-
8	Nue-112	GSR IR2-1-RF6-NU7-NU2-NU76-NU96	√	-	-	√	√	-
9	Nue-114	GSR IR2-1-RF6-NU7-NU3-NU82-NU97	√	-	√	√	-	-
10	Nue-229	GSR IR2-1-RF6-NU7-NU2-NU37-NU100	-	√	-	√	√	-
11	Nue-230	GSR IR2-1-Y17-NU2-NU5-NU6-NU7	-	√	-	√	√	-
12	Nue-115	GSR IR2-1-Y17-NU2-NU5-NU6-NU8	-	-	√	√	√	√

√-Response to high GY in the specific nutrient condition; - No response to high GY

4. Discussions

Development and wide adoption of GSR cultivars that can produce high and stable yields under less inputs have been considered as an essential step to achieve sustainable agriculture and maintain environmental quality [10]. Thus, greatly improved NuUE is one of the important green target traits in breeding GSR cultivars. However, plant breeders are facing at least three great challenges for improving NuUE. In the first place, improved NuUE is at most the secondary target trait in virtually all breeding programs because improved yield potential and adaptation to key major abiotic stresses in the target ecosystems would always be the number one priority in most breeding programs. For

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example, adequate tolerance to drought and salinity, in addition to the high yield potential, would be the priority traits for breeding programs for the rainfed ecosystem and saline areas. The second difficulty comes from the fact that NuUE is a complex trait of low heritability, which is affected most by the intrinsic large variation in soil fertility and type. The third challenge is apparent 'lack' of appropriate high NuUE donors because of fewer past efforts to identify rice germplasm accessions with high NuUE. As a result, there have been few well-documented breeding efforts for developing rice cultivars tolerant to nitrogen and phosphorus deficiencies [21, 22, 23, 24]. In this respect, results from this study provided at least partial answers to the three practical questions and demonstrated an innovative breeding method involving four consecutive rounds of selections under six different NuUE conditions for developing rice varieties with significantly improved NuUE.

4.1. Exploiting the 'hidden' genetic diversity in different sub-specific gene pools for improving NuUE

In the BC breeding procedure for developing ILs with improved NuUE, we used HAN, a *Geng* variety as the donor, to improve NuUE of a widely adaptable Xian variety, WTR1. WTR1 was released in Bangladesh as BRRI dhan69 in 2015 under the Green Super Rice (GSR) project for the 'Boro' conditions after six years of rigorous testing for grain yield and grain quality. Our results indicated that HAN did not seem to have a high NuUE but was able to contribute many useful genes/traits into WTR1, resulting in the development of many WTR1 ILs with greatly improved NuUE. This was consistent with our previous results that there is rich hidden genetic diversity in the different sub-specific gene pools that can be exploited for improving complex traits such as abiotic/biotic stress tolerances/resistances [23, 25, 26, 27,] using a modified BC breeding strategy [10, 23, 28]. This is not surprising because it is now known that different rice populations, particularly the two major subspecies, each contains large numbers of unique genes and alleles (haplotypes) that are absent or rare in other populations or subspecies ([19]. Thus, results from this study indicated that accessions of different subspecies could be a good source of useful genetic diversity for improving NuUE in rice.

4.2. Promising lines with high NuUE and selection efficiency for improved NuUE

In this study, we were able to identify many ILs, out of the 230 ILs, that had significantly higher yields (>30g in GY) than WTR1 under at least one nutrient deficiency condition, including 10 ILs under -NPK, 7 ILs under -N, 47 ILs under -P, 9 ILs under -NP, plus 12 promising ILs with significantly improved NuUE under more than one nutrient deficient conditions (Table 7). While this is consistent with other findings [27, 28] that high yielding varieties with high NuUE can be developed under NuUE deficiency conditions, it would be interesting to understand how the various types of selection schemes we adopted affect our selection efficiency for improving NuUE in rice. For the first round selection, we had approximately the same selection intensity under the IG, LI, and RF conditions, of which LI and RF were relevant to NuUE. Under the LI condition, we expected to select those plants which were tolerant to low NPK, because zero nutrient input was given to the LI field for 16 consecutive crop seasons before the selection. Surprisingly, the ILs selected under LI did not have improved yield under the -NP, -NPK, and -P conditions. Instead, these ILs had higher grain yields under the -N, 75N, and NPK conditions, when compared with those selected from IG and RF (Fig. 3). According to the soil fertility analyses, the available soil NPK contents of the LI field has reached a fairly constant low levels at 0.23N [KJn(%)], 24 P-Bray(mg/kg) and 1.19meq/100g) in an average of both the seasons of 2012 (WS and DS), 2013 (WS and DS), and 2014 (DS). Taking together, our results seemed to suggest these lines selected under LI had low P uptake ability but high P usability, and high N uptake and usability, which remain to be validated by more accurately, designed experiments. On the other hand, water deficiency was the primary factor liming rice yield performance under the RF condition where the first round selection was practiced. It was reported that plant water use efficiency is closely related to their NuUE because water uptake would facilitate nutrient uptake [24]. However, those ILs selected under RF showed relatively better GY under the -P and 75N condition and a much greater variation (72.1%) in GY and

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all measured traits across the six nutrient conditions than those selected from either IG or LI (Fig. 3). Furthermore, the IL (Nue-112) with highest NuUE was also selected from the RF conditions (Table 7). It suggested that drought tolerance and high NuUE in this population were apparently under independent genetic control. However, it is important to test if this is held generally true for other rice populations. For those ILs originally selected under IG (irrigated conditions), no overall yield advantages under the nutrient deficiency conditions were observed. Overall, the efficiency of the first round selection was RF > LI > IG for improving NuUE. Therefore, selecting of NuUE rice cultivars under rainfed situation is a prominent approach for the identification of superior rice cultivars.

4.3. Traits contributed to NuUE of rice

Our results indicated that high NuUE in rice is a very complex trait controlled by different genetic, physiological and molecular mechanisms. This conclusion was implicated by at least two observations. Firstly, the contributions of the three yield components to GY were relatively weak and varied considerably with each nutrient deficiency condition, though high and positive correlations between GY and BM were consistently observed across all nutrient deficient conditions. This was in contrast to previous reports that higher yields from enhanced grain filling ratio (GFR), spikelet number per panicle (SNP), number of panicles (NP) and TGW under nutrient deficiency conditions could be attributed to better nutrient absorption and uptake by plants after the application of fertilizer sources [29-34]. Earlier reports also indicated that grain number and weight primarily controlled by P and N [35, 36, 37], because P is more utilized in the formation of grain productivity and N influences tillering ability which contributed to higher yields through more panicles [38, 39]. Indeed, we observed that of the six nutrient conditions, NPK produced the highest PH, and the lowest PH was recorded in the -N condition. In the case of -NP condition, FLL, FLW and FLA traits were less and higher in the -P condition. TN was high in NPK, and low in 75N. Similarly, higher TN leading higher GY was also reported in rice [40]. Apparently, increased GY could be attributed to possible higher photo-assimilates and dry matter accumulation from increased leaf area in response to N fertilizers [41, 42]. TN, LL, LW, and LA were also reportedly significantly improved by the mining of nutrients, particularly N, through better root development, leading to improved translocation of carbohydrate capacity from expanded leaf rate [42, 43, 44]. Secondly, we observed that some ILs showed higher GY under one nutrient condition but lower GY in another (Table 5), while high GY of different ILs could be attributed to different combinations of yield components. For example, the high GY of Nue-112 under the NPK condition was mainly attributed to higher FGN, SN, PL, BM but not TGW because the TGW of Nue-112 was significantly lower than WTR1. However, its high GY under other conditions could be attributed to the simultaneous improvement of FGN, PL, BM, and TGW or the balance between them. Similarly, the high GY of Nue-115 could primarily be attributed to TGW, FLW (-NPK), FLW (-P), TGW (-N), respectively. Thus, the 230 ILs and their tremendous variation in NuUE provide useful materials for studying the genetic and physiological mechanisms of NuUE of rice, which is in progress.

5. Conclusions

Breeding of improved NuUE rice varieties is important for sustaining the rice production with depleting nutrient resources in the coming decades [45]. NuUE is a complex trait of low heritability, which is affected most by the intrinsic large variation in soil fertility and type [45]. Our study involved screening of inter-subspecific BC_1F_2 population with first round under RF, LI and IG and followed by a systematic phenotypic selection across six different nutrient conditions over four rounds that helped us to identify improved NuUE introgression lines. The study revealed that HAN did not possess a high NuUE but was able to contribute many useful genes/traits into WTR1, resulting in the development of many WTR1 ILs with greatly improved NuUE. These results confirm that there is rich hidden genetic diversity in the different sub-specific gene pools that can be exploited for improving complex traits such as abiotic/biotic stress tolerances/resistances using a modified BC breeding strategy. However, through this study, we could develop as many 230 ILs that is being utilized for studying the genetic and physiological mechanisms of NuUE of rice for different target NuUE related traits. The study demonstrated an efficient inter-subspecific BC breeding procedure with first round selection under the rainfed-drought condition followed by four generations of progeny testing for yield performances under six different nutrient conditions. Overall, the efficiency of the first round selection was RF > LI > IG for improving NuUE. Therefore, selecting of NuUE rice cultivars under rainfed situation is a prominent approach for the identification of superior rice cultivars. Identification of several promising introgression lines (Nue-115, Nue-114, Nue-112, Nue-229, and Nue-230) with improved NuUE would help rice breeding programs to utilize these materials for initiating new crosses and develop new breeding materials. These lines have now been promoted to the replicated multi-location yield trials for further testing for potential releases as new varieties in the target environments of several Asian and African countries.

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Conflicts of Interest: The authors declare that the research review was conducted in the absence of any commercial or economic associations that could be construed as a potential conflict of interest.

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