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Selection of Promising Homozygous Waxy Thermo-sensitive Genic Sterile (waxy-TGMS) Individuals from Three Segregating Rice (*Oryza sativa* L.) Populations

Nguyen Tuan Anh[1]*, Nguyen Thi Bich Hong[1], Nguyen Quoc Trung[2], Nguyen Thi Hoa[1], Vu Ngoc Lan[1], Tran Thi Thanh Xuan[1], Vu Van Son[1], Nguyen Van Hai[1], Nguyen Thanh Tung[3] & Tran Van Quang[1]

[1]Faculty of Agronomy, Vietnam University of Agriculture, Hanoi 12400, Vietnam
[2]Faculty of Biotechnology, Vietnam University of Agriculture, Hanoi 12400, Vietnam
[3]Institute of Crop Development, Vietnam University of Agriculture, Hanoi 12400, Vietnam

Abstract

The demand for glutinous rice for exporting encourages the breeding of new hybrid glutinous rice lines to meet the needed yield. Waxy-TGMS lines are principle materials to develop glutinous two-line hybrid rice, but have not been developed in Vietnam. This paper shows the selection of homozygous waxy-TGMS plants with advantageous agronomic traits from F_2 segregating populations to develop promising waxy-TGMS breeding lines. More than 3000 individuals from three F_2 populations derived from the crosses between non-waxy TGMS lines (*tms5* gene donor) and glutinous rice varieties (*wx* gene donor) were screened to picked up only 10 homozygous waxy-TGMS plants (*tms5/tms5 wx/wx*) with desired agronomic characters. F_3 seeds harvested from 10 desired F_2 individuals all had low amylose content like their waxy male parent variety and could be used to develop further into ten waxy-TGMS breeding lines.

Keywords

TGMS, glutinous, two-line hybrid rice, marker-assisted selection

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Correspondence to
Nguyen Tuan Anh
nguyentuananh@vnua.edu.vn

ORCID
Nguyen Tuan Anh
https://orcid.org/0000-0002-7188-2333

Introduction

Glutinous rice is a very important and preferred ingredient in cooking many kinds of traditional desserts in Vietnam. It is also an important export product, accounting for up to 10% of the total Vietnamese rice exports (MARD, 2024). To cope with the reduction in Vietnamese rice growing areas (Nhu Mai, 2021) and rising population numbers, increasing rice yield is of the utmost necessity. The most effective way to increase rice yield is by using hybrid varieties to exploit heterosis. Although many hybrid rice varieties

have been created in Vietnam since 2000, no hybrid glutinous rice has been developed (Tran *et al*., 2021).

Compared to three-line hybrid rice, which has a higher labor cost and longer time for hybrid seed production, the two-line hybrid rice system is simpler, costs less, and very popular in Vietnam. For instance, among the 19 hybrid rice varieties developed in Vietnam during the period of 2002-2013, 11 varieties were two-line hybrid rice (Pham *et al.*, 2015). Using the *tms5* genes from non-waxy TGMS E15S (maternal line of the two-line hybrid rice HQ19 (Tran *et al.*, 2014)) and TUS (a developing maternal TGMS line) crossed with two inbred glutinous rice varieties, we aimed to create waxy-TGMS lines, which could be used as maternal lines for the upcoming two-line waxy hybrid rice. This paper describes the selection for the morphological phenotypes and target genotypes on F_2 segregating populations of the mentioned crosses to identify excellent homozygous waxy-TGMS individuals in order to obtain candidate F_3 families for further breeding.

Materials and Methods

Plant materials

This study used two sources of TGMS, which were E15S and TUS. E15S is an aromatic TGMS line with a critical sterility-inducing point (CSIP) at 24°C (Tran *et al.*, 2013). This line carries TGMS gen *tms5* as described by Pham *et al.* (2015). While TUS is a recently developed line and carries *tms5* as well. Two sources of waxy genes (*wx*) were obtained from the Vietnamese Agricultural Genetic Institute, "Nếp 97" (N97), a popular waxy rice variety in Northern Vietnam, and "Nếp Thái" (NTL), a popular glutinous rice in Southern Vietnam. Three crosses (E15S × N97), (E15S × NTL), and (TUS × N97) were carried out in the spring of 2019. F_1 plants were grown in the autumn of 2019, and the seeds of the F_2 offspring were grown during the 2020 spring season in Gia Lam, Ha Noi as materials for this study.

Seedlings nursery

The seeds of each line were soaked in fresh water at 28°C for 72 hours, then incubated for 24 hours at 28°C for seed germination. The germinated seeds were sown on a seedbed, pre-fertilized by 10g urea (CH_4N_2O) and 10g potassium chloride (KCl) per m^2, with a density of 1 seeds cm^{-2}. When the seedlings had approximately six leaves, they were transplanted to the experimental field.

Experimental design

Three F_2 populations of (E15S × N97) [1450 plants], (E15S × NTL) [700 plants], and (TUS × N97) [1200 plants] were transplanted in a light-clay paddy field with plant density of 27 plants m^{-2}, into three successive blocks without replication. Two rows of each of the four parental lines (E15S, TUS, N97, and NTL) were transplanted between the blocks.

N-P-K fertilizer was applied to the field equal to 70kg N – 70kg P_2O_5 – 70kg K_2O per hectare. Half of the total fertilizer was mixed into the soil during soil preparation (i.e before transplanting), and the remaining half of the fertilizer was sprinkled on the paddy field 14 days after transplanting.

Selection of promising individuals in the F2 segregating populations

Three stages of selection were used to select candidates for potential homozygous TGMS plants from the F_2 populations. Firstly, the F_2 plants were screened for having "advantageous" morphology by meeting pre-built ideotype criteria for important agronomic traits. Secondly, advantageous individuals were screened for homozygous waxy locus (*wx/wx*). Finally, the homozygous waxy plants were genotyped to pick up homozygous (*tms5/tms5)* individuals.

Morphological agronomic trait measurements

The morphological trait measurements were carried out based on the Standard Evaluation System (SES) for Rice Standard (IRRI, 2014). The GD of a plant was the period from the date of seed germination to the date of heading. The NL was marked and counted. The SH was measured from the ground surface to the top tip of the main panicle. TA was the angle between the main tiller and its adjacent tiller measured by a protractor, then categorized as code 1 (< 15°);

3 (15-30°); 5 (30-45°); 7 (45-60°); and 9 (> 60°). The length from the leaf knee of the flag-leaf blade (FLL), maximum width of the flag-leaf blade (FLW), and the angle between the flag-leaf mid rib and main stem (FLA) were measured by a ruler and protractor, respectively. TNP was the sum of the plant panicles having more than 10 spikelets. The number of spikelets per panicle (SPP) was counted on the main panicle (the first heading and usually the biggest panicle). The weight (m) of the number of filled seeds of the main panicle (n) was used to estimate the 1000-grain weight (M1000) as M1000 = m/n*1000 (g). Finally, the potential yield (PY) was estimated as PY = TNP * SPP * M1000 / 1000 (g plant^{-1}).

Overview of the variability of a population by quantitative analysis

The variability of the quantitative morphological traits in each F_2 segregating population were estimated by population parameters, namely average, standard deviation (SD), variance (VP), max, min, and 95% confident interval of the mean (95% CI), of the samples of 148, 131, and 118 individuals obtained from F_2 (E15S × N97), F_2 (TUS × N97), and F_2 (E15S × NTL), respectively. Broad-sense heritability (H^2) of the morphological traits was estimated using ***Equation 1:***

$$(A) \qquad VE_{F2} = {}^{(VP_{P1} + VP_{P2})}/_{2}$$

$$(B) \qquad VG_{F2} = VP_{F2} - VE_{F2}$$

$$(C) \qquad H^2 = \frac{VG_{F2}}{VP_{F2}}$$

Equation 1. The three steps of estimating broad sense heritability. (A) The environmental variance component (VE_{F2}) was estimated by averaging the two phenotypic variances of the two parental inbred lines of each segregating population, which had no genetic variance ($VG_{F2} = VG_{F2} = 0$). (B) The genetic variance of a segregating population (VG_{F2}) is equal to the difference between the phenotypic variance (VP_{F2}) and environmental variance (VE_{F2}). (C) Broad sense heritability is the proportion of (VG_{F2}) to (VP_{F2}).

Morphological selection (1st selection)

Morphological selection was based on the ideotype that originated by Yuan (2001) and then modified by the authors based on the variability of the segregating populations: (1) the growth duration (GD) ranges from 100-130 days in the spring season; (2) the number of leaves on the main tiller (NL) is more than 13; (3) the shoot height (SH) is more than 80cm; (4) the tiller angle (TA) less than 30°; (5) the flag-leaf length (FLL) is more than 35cm; (6) the flag-leaf width (FLW) is more than 2cm with a V-shape; (7) the flag-leaf angle (FLA) is less than 30° and the tips of the top three leaves are higher than the panicle; (8) the total number of panicles (TNP) is more than eight; and (9) it has the potential yield (PY) of at least 37 g plant^{-1} (equal to the yield greater than 10 tons ha^{-1} with a density of 27 plants m^{-2}).

Selected plants with the sign of stigma exertion (the typical phenotype of homozygous TGMS segregants or of some offspring between waxy and non-waxy rice) were covered by a plastic tube to a height of 40cm above their highest panicle to prevent out-crossing. The seeds of selected individuals were harvested and stored separately for the 2nd selection as well as for develop F_3 family if needed.

Homozygous waxy plants selection (2nd selection)

Low amylose content in rice grains (waxy grains) are controlled by a recessive allele *wx* of the waxy locus (Chen *et al*., 2008). Twenty F_3 seeds from each selected F_2 individual were de-husked and then broken at their middle before being stained by an I_2KI 0.5% solution. After 5 minutes of staining, the numbers of red brown (waxy) grains and dark blue (non-waxy) grains (Zhang *et al.*, 2018) were recorded, and the waxy locus genotype of the F_2 plant could be concluded based on the proportion of grain colors (**Figure 1**). A homozygous waxy F_2 individual (*wx/wx*) will have 20 red brown stained F_3 seeds (**Figure 1**, left), a homozygous non-waxy F_2 individual (*WX/WX*) will have 20 dark blue stained F_3 seeds (**Figure 1**, right), and a heterozygous plant of the waxy locus (*WX/wx*) will have both red brown and dark blue stained F_3 seeds (**Figure 1**, center).

Figure 1. Pictures of 0.5% I_2KI stained grain of waxy (wx/wx) plant - 100% red brown grains, heterozygous (WX/wx) plants - mix of red brown and dark blue grains, and non-waxy (WX/WX) plants - 100% dark blue grains plants among the F2 populations.

MAS for homozygous TGMS plant (3rd selection)

Homozygous waxy plants, which had been determined from the 2nd selection, were genotyped for the *tms5* locus by marker C356-1, as referred to Wang *et al*. (2003). Total genomic DNA of each plant was extracted from dried leaves using the "potassium acetate" protocol with minor modifications (Dellaporta *et al*., 1983). PCR amplifications were carried out on a SimpliAmpTM Thermal Cycler (Applied Bissystems™), in a 10-µL volume PCR reaction, which included 1µL of approximately 50 ng mL^{-1} DNA template, 5µL of 2X reaction buffer (PCR Master Mix 2X, Intron®, Korea), 1µL of 2 µM forward primer (5' ATTTTGGTTGCGCATTAGAGG 3'), 1µL of 2 µM reverse primer (5' GAAATATGCCAAGTACGGAGGAT 3'), and 2.0µL of distilled water. The PCR program was started by one cycle at 94°C for 5min, continued by 40 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 45s, and finally ended by one cycle at 72°C for 10min. PCR products were separated in 3% agarose gel mixed with 1X RedSafe®, Intron, Korea (0.5µL RedSafe per mL agarose gel) under electrophoresis at a 75-mV current for approximately 60min. DNA band detection was carried out under UV light at 450nm, and a picture of the gel plate was taken by a camera at a resolution of 12 megapixels. A *tms5*-homozygous plant (S) will have a DNA band at size 435bp, while a *TMS5*-homozygous plant (F) will have a DNA band at 418bp (Wang *et al*., 2003). Heterozygous plants (H) will have bands at both 435bp and 418bp.

Amylose content measurement

The amylose content in the rice grain was predicted by spectrophotometry. Dried grain samples were ground to a fine powder. A 100-mg sample of the powder was collected and poured into a conical flask, to which 1mL of 95% ethanol and 9mL of 1M NaOH were added. The mixture was heated in boiling water for 10min to disperse the starch. 0.5 mL of the cooled starch solution was transferred to a new flask, to which 9.2mL water, 0.1mL of 1N acetic acid, and 0.2 ml of 0.2% iodine solution were added to make exactly 10mL of solution. Spectrophotometer measurements were made at 620nm after the above starch-iodine solution was incubated for 30min. The absorbance unit (AU) of a sample was projected to a linear regression equation of the AU of five standard rice flour samples, which have known amylose contents varying from 0-30%, to predict the amylose content of the sample (TCVN 5716-2:2017 ISO 6647-2).

Statistical analysis

Mean, SD, VP, max, min, 95% CI and statistical comparison of all measurements were carried out by STAR (STAR, 2013). Following analysis of variance (ANOVA), pairwise comparison of means was done with Fisher-LSD test at 5%.

Results

Morphological variability of the F2 segregating populations

Two paternal waxy lines, N97 and NTL, had longer growth durations (GDs) than the two TGMS lines, E15S and TUS. The GDs of N97 and NTL were 109 days and 118.3 days, respectively, longer than the 102.9 days of E15S and 99.5 days of TUS (P <0.05) (**Table 1**). The average GDs of the F_2 populations were not different from their maternal lines but different from their paternal lines (**Table 1**), which indicated that the GDs of the F_2 individuals were more influenced by their maternal parent (shorter GD) rather than their paternal parent (longer GD). In the F_2 populations, the individuals with the greatest GD values were all longer than their paternal parents' GD by at least 15 days, while the ones with the minimum GD values were rarely shorter than that of their maternal parent (**Table 1**). The broad-sense heritability (H^2) values of this trait in the three F_2 populations were all greater than 0.6, which gave a signal of feasible selection for GD.

The NL values of the segregating populations were all located between the NL of their two parents. While the F_2 populations' average SH values were consistently lower than 70cm, which were just like their parents' SH (**Table 1**). The heritability values of two traits were all lower than 0, and would not be used in morphological selection.

The heritability of TA varied from 0.5 to 0.7 in the F_2 populations (**Table 1**), indicating that the trait could be selected for effectively from segregating populations. TA varied from scores of 1 to 3 (i.e. less than 30°) among all three populations and fully met the desired TA without selection (**Table 1**).

FLL, FLW, and FLA had huge phenotypic variation among the segregating populations. The max and CI95% values of these traits in the three samples of the F_2 populations indicated that the selection of FLL, FLW, and FLA as ideotype checklists was possible. However, their heritability fluctuated a bit, varying from 0.3 (weak) to 0.7 (strong) among the three F_2 populations (**Table 1**). These traits should be selected for in further generations, not in the F_2 populations.

Among the parental lines, the total number of panicles (TNP) of the two paternal lines (21.2 panicles for N97 and 30.2 panicles for NTL) were significantly higher than those of the maternal TGMS lines (14.9 panicles for E15S and 14.3 panicles for TUS) (**Table 1**). However, the capacity of producing panicles of an F_2 population highly overcame their parents. The maximum TNP values of an individual in F_2 (E15S × NTL), F_2 (E15 × N97), and F_2 (TUS × N97) were 68 TNP, 65 TNP, and 54 TNP, respectively. TUS had the highest number of spikelets per panicle (SPP) (approximately 148.3 spikelets) while NTL had the lowest SPP (just 64.2 spikelets) (**Table 1**). All three F_2 populations had an average SPP higher than that of their paternal line (i.e. N97 or NTL) but lower than the SPP of their maternal line (i.e. E15S or TUS) (**Table 1**). The 1000 grain weights (M1000) of the paternal lines (26.8g for N97 and 25.3g for NTL) were moderately higher than those of the two maternal lines (22.2g for E15S and 23.1g for TUS). There were high levels of variation of M1000 in the F_2 populations, ranging approximately from 17.1 to 32.7g in F_2 (E15S × N97), from 15.7 to 35g in F_2 (E15S × NTL), and from 17.3 to 38.2g in F_2 (TUS × N97) (**Table 1**). The population F_2 (TUS × N97) appeared to have the highest average M1000 (approximately 28g), compared to 24.4g of F_2 (E15S × N97) and 24.1g of F_2 (E15S × NTL) (**Table 1**). Like the yield component traits (i.e. TNP, SPP, and M1000), the measurement of potential yield (PY) of an F_2 population seemed influenced by both of its parents. F_2 (TUS × N97), which had the highest PY (approximately 68g), compared to the PY values of F_2 (E15S × NTL) (roundly 61g) and of F_2 (E15S × N97) (just 52.6g), also had the highest PY sum of its parents (48.9g for TUS + 43.2g for N97) comparted to the sum of the parents of F_2 (E15S × NTL) (33.6g for E15S + 49.9g for NTL) and the parents of F_2 (E15S × N97) (33.6g for E15S + 43.2g for N97) (**Table 1**). The heritabilities (H^2) of the four traits of TNP, SPP, M1000, and PY were all greater than 0.6 for the three F_2 populations, except for H^2 = 0.4 of SPP in F_2 (TUS × N97), which revealed that the traits could be selected effectively.

Morphological selection on F2 segregating populations

Selection for GD was carried out based on the GD of E15S, which had been confirmed to be around 67 days in the autumn season in 2012 (Tran *et al.*, 2013). In the 2020 spring season, the GD of E15S was 106 days, and any individuals with a GD greater than 136 days would be ignored from morphological selection because of having a too long growth duration, compared to E15S. A few plants had GD shorter than 106 days. These plants were tiny and had no good characteristics of yield components. Selection on FLL, FLW and FLA, TNP, SPP, M1000, and PY was straightforward; individuals with measurements meeting the ideotype's criteria were selected. There were 54, 11, and 77 plants which were selected in F_2 (E15S × N97), F_2 (E15S × NTL), and F_2 (TUS × N97), respectively. The selected plants were gathered into "selected groups". The selected groups had average TNP, average SPP, and average YP much bigger than the average values of the F_2 populations themselves, except for the trait of M1000 (Table 1). The TNP values of the selected groups were 45%, 56%, and 35% higher than those of F_2 (E15S × N97), F_2 (E15S × NTL), and F_2 (TUS × N97), respectively. At that time, the SPP values of the selected groups were 21%, 55%, and 0% higher than those of F_2 (E15S × N97), F_2 (E15S × NTL), and F_2 (TUS × N97), respectively. The M1000 values of the selected groups from F_2 (E15S × N97) and F_2 (E15S × NTL) were like their non-selected F_2 populations (24.6g vs. 24.4g; and 23.3g vs. 24.1g, respectively). The M1000 of the selected group from F_2 (TUS × N97) was lower than the non-selected F_2 (TUS × N97) (24.9g vs. 28g). The average PY of the selected group from F_2 (E15S × NTL) was 145.4 g plant^{-1}, which was much higher than the PY of the selected groups from F_2 (E15S × N97) at 94.8 g plant^{-1} and from F_2 (TUS × N97) at 81.2 g plant^{-1} (**Table 1**).

Selection for homozygous waxy plants from morphologically selected plants

The selection of waxy plants, which was carried out on a total of 142 morphologically selected plants from the three F_2 populations, showed that there were 39 glutinous (*wx/wx*) individuals, 67 heterozygous (*WX/wx*) individuals, and 36 non-glutinous (WX/WX) individuals (Table 2), which fitted well to the expected 1:2:1 ratio in the segregating population of single-gene traits. The chi-square test for the goodness of fitness ($\chi2$) was equal to 0.57 ($P > \chi2 = 0.75$), much lower than the value 5.99 of $\chi2$standard (0.05, df=2) (**Table 2**). The significant difference in the goodness of fit between the observed phenotypic ratio and the expected phenotypic ratio could be recorded in each selected group from each F_2 population with a smaller number of individuals. The $\chi2$ for each selected group of F_2 (E15S × N97), F_2 (E15S × NTL), and F_2 (TUS × N97) were very tiny, approximately 0.33 ($P > \chi2 = 0.85$), 0.27 ($P > \chi2 = 0.87$), and 0.22 ($P > \chi2 = 0.90$), respectively (detailed calculations not shown). These results revealed that the waxy gene and the genes controlling the selective morphological traits (GD, TNP, SPP, M1000, and YP) showed independent assortment.

Molecular-assisted selection (MAS) for homozygous TGMS-plants

Genotyping the tms5 locus of 39 homozygous waxy plants by marker C365-1 revealed that 10 plants likely had the homozygote tms5 genotype (tms5/tms5 - S), 19 plants had heterozygous genotype (TMS5/tms5 - H), and 10 plants had the homozygote TMS5 genotype (TMS5/TMS5 - F) (**Figure 2** and **Table 3**).

The observed ratio of the three genotypes (S, H, F) among the entire three F_2 populations was 10:19:10, which closely fit the expected 1:2:1 ratio in a segregating population of single-gene traits. The chi-square test for the goodness of fit showed that the $\chi2$ value was equal to 0.03 ($P > \chi2 = 0.98$), much lower than the value 5.99 of $\chi2$standard (0.05, df=2) (**Table 3**).

Ten homozygous waxy-TGMS individuals were selected from F_2 (E15S × N97) and F_2 (TUS × N97) but no individual was selected from F_2 (E15S × NTL). The PYs of the candidate plants ranged from 40.1 g plant^{-1} to 278 g plant^{-1} (**Table 4**). Among them, six individuals had a potential yield significantly higher than both their parents, while four individuals had PYs not significantly different from their parents (**Table 4**). Noticeably,

Table 1. Morphological survey on the three F_2 populations, their mother (E15S or TUS), their father (N97 or NTL), and the three groups of advantageous individuals, which were selected from the F_2 populations

Traits	E15S			N97				F_2 (E15S × N97)							Seleted plants				
	n	Mean	SD	n	Mean	SD	sig	n	Mean	SD	Min	Max	95% CI	H^2	n	Mean	SD	Min	Max
Growth duration (GD)	15	102.9	3.3	10	109.0	1.6	*	148	105.3	6.2	98.0	125.0	(95, 115.5)	0.8	-	-	-	-	-
Shoot height (SH)	15	69.4	3.7	10	61.4	8.6	*	148	67.1	7.3	45.0	84.0	(55, 79.1)	0.2	-	-	-	-	-
No. of leaves on main stem (NL)	15	15.2	1.0	10	15.8	0.4	ns	148	15.1	0.7	13.0	16.0	(13.9, 16.3)	-0.2	-	-	-	-	-
Tiller angle (TA)	15	1.8	0.4	10	2.5	0.5	*	148	2.4	0.7	1.0	3.0	(1.3, 3.5)	0.5	-	-	-	-	-
Flag-leaf length (FLL)	15	29.5	5.3	10	24.5	5.0	*	148	27.0	6.0	13.0	51.0	(17.1, 37)	0.3	-	-	-	-	-
Flag-leaf width (FLW)	15	1.7	0.2	10	1.9	0.1	*	148	1.8	0.2	0.6	2.5	(1.4, 2.2)	0.5	-	-	-	-	-
Flag-leaf angle (FLA)	15	19.6	4.7	10	14.0	3.6	*	148	17.7	5.6	4.0	30.0	(8.5, 26.9)	0.4	-	-	-	-	-
Total number of panicles (TNP)	15	14.9	1.8	10	21.2	5.5	*	148	23.5	11.5	6.0	65.0	(4.5, 42.5)	0.9	54	34.1	16.1	9.0	78.0#
Seeds per panicle (SPP)	15	101.5	13.4	10	76.2	16.3	*	148	88.2	26.4	21.7	173.0	(44.6, 131.9)	0.7	54	106.6	41.5	51.0	225.0#
Weight of 1000 grains (M1000)	15	22.2	0.5	10	26.8	1.6	*	148	24.4	2.5	17.1	32.7	(20.2, 28.6)	0.8	54	24.6	3.5	18.5#	40.4#
Potential yield (PY)	15	33.6	5.8	10	43.2	13.6	ns	148	52.6	36.8	6.1	216.1	(-8.2, 113.5)	0.9	54	94.8	85.4	24.5	468.5#
Traits	E15S			NTL				F2 (E15S × NTL)							Selected plants				
	n	Mean	SD	n	Mean	SD	sig	n	Mean	SD	Min	Max	95% CI	H^2	n	Mean	SD	Min	Max
Growth duration (GD)	15	102.9	3.3	10	118.3	4.3	*	118	107.3(2)	6.0	100.0	133.0	(97.3, 117.4)	0.6	-	-	-	-	-
Shoot height (SH)	15	69.4	3.7	10	59.2	6.2	*	118	67.1(2)	7.0	45.0	82.5	(55.4, 78.8)	0.5	-	-	-	-	-
No. of leaves on main stem (NL)	15	15.2	1.0	10	15.9	0.3	ns	118	15.2(2)	0.7	13.0	16.0	(14, 16.4)	-0.2	-	-	-	-	-
Tiller angle (TA)	15	1.8	0.4	10	1.7	0.5	ns	118	1.7(1)	0.8	1.0	3.0	(0.4, 2.9)	0.7	-	-	-	-	-
Flag-leaf length (FLL)	15	29.5	5.3	10	27.2	3.4	ns	118	27.9	7.0	12.0	49.0	(16.3, 39.6)	0.6	-	-	-	-	-
Flag-leaf width (FLW)	15	1.7	0.2	10	1.8	0.1	ns	118	1.7	0.2	1.2	2.1	(1.4, 2)	0.3	-	-	-	-	-
Flag-leaf angle (FLA)	15	19.6	4.7	10	9.5	3.4	*	118	18.9(2)	6.5	2.0	50.0	(8.2, 29.7)	0.6	-	-	-	-	-
Total number of panicles (TNP)	15	14.9	1.8	10	30.2	8.7	*	118	26.8(1)	12.9	6.0	68.0	(5.4, 48.2)	0.8	11	41.8	16.0	23.0	66.0#
Seeds per panicle (SPP)	15	101.5	13.4	10	64.2	18.2	*	118	89.6(2)	27.4	32.0	188.0	(44.1, 135.1)	0.7	11	139.3#	54.7	64.0	240.0#
Weight of 1000 grains (M1000)	15	22.2	0.5	10	25.3	1.3	*	118	24.1	2.7	15.7	35.0	(19.6, 28.6)	0.9	11	23.2	2.4	20.7	28.6
Potential yield (PY)	15	33.6	5.8	10	49.9	24.7	*	118	61.0	41.3	6.0	218.6	(-7.5, 129.5)	0.8	11	145.4#	100.4	16.3	348.5#

Traits	TUS			N97				F_2 (TUS × N97)						Selected plants				
	n	Mean	SD	n	Mean	SD	sig	n	Mean	SD	Min	Max	95% CI	H^2	n	Mean	SD	Min	Max
Growth duration (GD)	15	99.5	3.1	10	109.0	1.6	*	131	103.4(2)	5.3	98.0	129.0	(94.5, 112.2)	0.8	-	-	-	-	-
Shoot height (SH)	15	81.2	5.1	10	61.4	8.6	*	131	61.7(1)	7.4	45.0	90.0	(49.4, 74)	0.1	-	-	-	-	-
No. of leaves on main stem (NL)	15	14.6	1.5	10	15.8	0.4	*	131	15.6(1)	0.6	13.0	16.0	(14.6, 16.6)	-2.5	-	-	-	-	-
Tiller angle (TA)	15	1.2	0.4	10	2.5	0.5	*	131	2.1	0.7	1.0	3.0	(0.9, 3.3)	0.6	-	-	-	-	-
Flag-leaf length (FLL)	15	30.0	3.8	10	24.5	5.0	*	131	27.9	5.9	16.0	41.0	(18.2, 37.6)	0.4	-	-	-	-	-
Flag-leaf width (FLW)	15	1.5	0.1	10	1.9	0.1	*	131	1.9(1)	0.2	1.2	2.5	(1.6, 2.3)	0.7	-	-	-	-	-
Flag-leaf angle (FLA)	15	14.9	4.6	10	14.0	3.6	ns	131	14.1	5.5	5.0	30.0	(5, 23.2)	0.4	-	-	-	-	-
Total number of panicles (TNP)	15	14.3	3.6	10	21.2	5.5	*	131	22.2	9.8	6.0	54.0	(6, 38.4)	0.8	77	30.0	12.7	12.7#	75.0#
Seeds per panicle (SPP)	15	148.3	23.8	10	76.2	16.3	*	131	105.5(1)(2)	26.7	46.0	194.0	(61.3, 149.7)	0.4	77	106.9	41.0	41.0#	308.0#
Weight of 1000 grains (M1000)	15	23.1	1.1	10	26.8	1.6	*	131	28(1)	4.4	17.3	38.2	(20.8, 35.2)	0.9	77	24.9	2.9	19.1#	37.1#
Potential yield (PY)	15	48.9	14.0	10	43.2	13.6	ns	131	68.0	41.8	12.4	276.5	(-1.2, 137.2)	0.9	77	81.2	49.8	49.8	241.4#

Note: "n" implies the number of surveyed individuals; "SD", "min" and "max" imply the value of the standard deviation, minimum value and maximum value of "n" survey individuals; "95% CI" implies the 95% confident interval of the mean; "H^2" implies the heritability with bolded values being greater than 0.5; (*) implies a significant difference between the father and mother while "ns" implies insignificance; (1) and (2) implies significant difference of the mean of a F_2 sample from its mother and from its father, respectively; "#" implies the value is out of "95% CI" the mean of relevant F_2 sample. All statistical comparison is Fisher-LSD at significant level of 5%.

Table 2. Summary of the waxy (wx) genotypes of the selected plants in the F_2 populations.

Genotypes	F_2 population			Total observed ratio (O)	Expected ratio (E)
	(E15S × N97)	(E15S × NTL)	TUS × N97)		
wx/wx (N)	16	2	21	39	1
WX/wx (H)	24	6	37	67	2
WX/WX (T)	14	3	19	36	1
Total	54	11	77	142	$\chi^2 = 0.57$*
					$P(\chi^2 \geq 0.57) = 0.75$

Note: An asterisk in the chi-square value (χ^2) indicates that the observed genotypic ratio (O) was significantly fitted to the expected genotypic ratio (E) at a 0.05 significance level, with df = 2.

the total number of panicles (TNP) seemed to play a vital role in PY. Six of the plants that had the highest PY also had the highest number of TNP among the 10 selective plants. However, while the average TNP of the selected plants from F_2 (E15S × N97) was higher than that of F_2 (TUS × N97) (53 panicles vs 34.1 panicles, P = 0.052), the average of M1000 of the group from F_2 (TUS × N97) was higher than that from F_2 (E15S × N97) (27.4g vs 21.4g, P = 0.026).

Because the amylose content in rice grains is not completely regulated by the waxy gene, it was assayed for the F_3 grains obtained from the 10 candidate F_2 individuals to ensure the effectiveness of the 2nd selection. The assay revealed that the amylose contents of the grains of all the F_2 candidates were much lower than that of the non-waxy variety KD18 (a standard non-waxy variety) and as low as that of the waxy variety N97 (a standard waxy variety). The amylose content of the F_3 grains varied from 5.3% to 9.8%, while that of N97 and KD18 were 6.5% and 24.3%, respectively (**Table 4**).

Discussion and Conclusions

Breeding waxy-TGMS lines is essential to develop glutinous two-line hybrid rice. Three F_2 populations derived from maternal non-waxy TGMS rice and paternal waxy rice were created to select candidates of homozygous waxy-TGMS individuals with excellent agronomic phenotypes. The selection strategy included three stages: the 1st selection for the morphological phenotypes, 2nd selection for the homozygous waxy genotype, and 3rd selection for the homozygous TGMS genotype.

Firstly, the individuals with excellent agronomic traits were picked out from the segregating populations based on a table of criteria for important agronomic quantitative traits. Biometrical analyses for the variability and broad-sense heritability of each trait were estimated from a subset of random individuals of each population to give the range of each trait and the potential gain of selection. As a consequence, four characters with very high broad-sense heritability that were related to grain yield, namely TNP, SPP, M1000, and PY, were used to select 142 outstanding individuals (**Table 1**). Among them, 54 plants were obtained from F_2 (E15S × N97) (3.2% selection), 11 plants were from F_2 (E15S × NTL) (1.6% selection), and 77 plants were from F_2 (TUS × N97) (6.4% selection). These selection intensities were very low (< 5% on average), which could lead to very high realized heritability in the selection for M100 and PY (Vir & Singh, 2005) and also for TNP and SPP, which were highly positive, corresponding to PY (Senapati *et al.*, 2009).

After selection on morphology, 142 selected plants were genotyped at the waxy locus by segregation analysis. Thirty-nine homozygous waxy individuals, 36 homozygous non-waxy plants, and 67 heterozygous plants were identified. This genotypic ratio was highly fitted to the expected ratio of 1:2:1 (**Table 2**), which implied that the waxy gene independently assorted from the segregation of the morphological traits. Segregation analyses were carried out by calculating the ratio of waxy or/and non-waxy endosperm of twenty F_3 grains derived from an F_2 plant (see the methodology section). There was a concern about the accuracy of this method related to the size of the grain samples that needs to be discussed. In theory, with a "big enough" F_3 grain sample, homozygous waxy or non-waxy F_2 plants will form only 100% waxy or 100% non-waxy F_3 grains while heterozygous waxy plants will produce 25% waxy endosperm (*wx/wx/wx*, 3n) and 75% non-waxy grain (WX/-/-, 3n). By taking the 20-grain F_3 samples, it was impossible to inaccurately conclude that a homozygous waxy or non-waxy individual was a heterozygous one (P = 0), or a heterozygous plant was homozygous waxy plant (P = 0), but a heterozygous plant could be concluded as homozygous non-waxy plant (P = 0.31%, which is the rate of error) (calculated using the online binomial calculator https://stattrek.com/online-calculator/binomial.aspx). This error rate is similar to that of a MAS using a marker that is 0.3cM away from a target gene (i.e. very tightly linked). The size of the F_3 grain samples could be bigger to get a lower rate of error, but this costs more in terms of labor and time for analysis.

Table 3. Summary of the tms5 genotypes of the selected waxy plants in the F_2 populations

Genotypes	F_2 population of			Total observed ratio (O)	Expected ratio (E)
	(E15S × N97)	(E15S × NTL)	(TUS × N97)		
tms5/tms5 (S)	4	0	6	10	1
TMS5/tms5 (H)	9	1	9	19	2
TMS5/TMS5 (F)	3	1	6	10	1
Total	16	2	21	39	$\chi^2 = 0.03$*
					$P(\chi^2 \geq 0.03) = 0.99$

Note: An asterisk in the chi-square value (χ^2) indicates that the observed genotypic ratio (O) was significantly fitted to the expected genotypic ratio (E) at a 0.05 significance level, with df = 2.

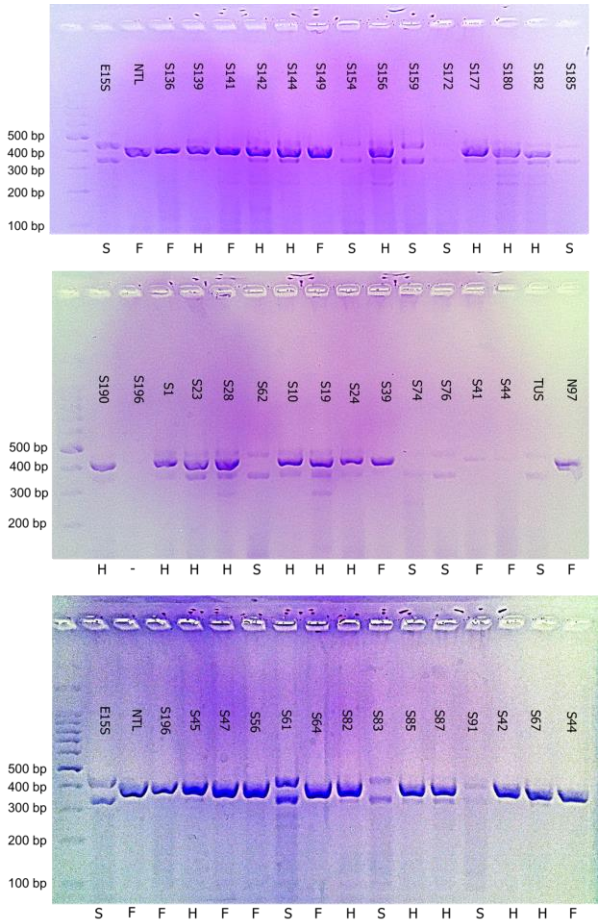

Figure 2. Electrophoresis images of the PCR products of marker C365-1 with the DNA templates of the four parent lines (a) and of some of the morphologically selected individuals from the F_2 populations (b). The text inside the pictures labels the DNA templates, while the text below the image implies the genotype of plants (S implies sterile, H implies heterozygous, and F implies fertile plants). Band 435 bp was specific for the C365-1 allele linked to recessive tms5, while the 418-bp band was specific for the C365-1 allele linked to TMS5. Noticeably, there was a shorter unspecific band (approximately 370 bp) in the TGMS plants for an unknown reason.

The amylose contents of the 10 selected waxy-TGMS plants, which varied from 5.6% to 9.8%, were all much lower than the 24.3% of the checked non-waxy grains of KD18, which could be used to prove the reliability of the segregation analysis. The TGMS plants were identified by the marker C365-1, which is 1.04 cM away from the *tms5* locus (Wang *et al*., 2003; Yang *et al*., 2007). While the putative genomic region of *tms5* ranged from 6,397,342 to 6,399,236 in

Table 4. Characteristics of the advantageous waxy-TGMS F_2 plants.

No.	ID	Origin	Waxy/ non-waxy	*tms5 (S/H/F)*	TNP (panicles)	SPP (spikelet)	M1000 (g)	PY (g plant^{-1})	95% confident interval of PY (g plant^{-1})	Amylose content of F_3 seeds, derived from selected F_2 individual (%)
1	20XS154	E15S × N97	waxy	S	67	225	18.5	278.3$^{(1)(2)}$	-	5.6
2	20XS159	E15S × N97	waxy	S	69	164	23.4	265.1$^{(1)(2)}$	-	6.5
3	20XS185	TUS × N97	waxy	S	62	105	37.1	241.4$^{(1)(2)}$	-	6
4	20XS74	E15S × N97	waxy	S	37	136	22	110.7$^{(1)(2)}$	-	6.1
5	20XS76	E15S × N97	waxy	S	39	100	21.8	85$^{(1)(2)}$	-	7.4
6	20XS172	TUS × N97	waxy	S	39	87	24.8	84$^{(1)(2)}$	-	9.8
7	20XS61	TUS × N97	waxy	S	24	105	26.4	66.5	-	5.3
8	20XS62	TUS × N97	waxy	S	22	94	27	55.8	-	5.4
9	20XS83	TUS × N97	waxy	S	32	60	24.5	47	-	6
10	20XS91	TUS × N97	waxy	S	26	63	24.5	40.1	-	6.7
	E15S	Parental	non-waxy	S	14.9	101.5	22.2	33.6	21.2 - 46.1	-
	TUS	Parental	non-waxy	S	14.3	148.3	23.1	48.9	18.9 - 78.9	-
	N97	Parental	waxy	F	21.2	76.2	26.8	43.2	12.5 - 73.9	6.5
	KD18	Checked	non-waxy							24.3

Note: (1) and (2) imply that the PY of an individual was significantly different from the mean of the PY of their maternal line (E15S or TUS) and paternal line (N97), respectively. The grains of N97 and of KD18 were used as the control for waxy and non-waxy, respectively.

IRGSP-1.0 (Zhou *et al*., 2016), the marker C365-1 is located in the region from 6,710,686 (start of forward primer) to 6,711,303 (start of reverse primer), therefore producing an expected PCR product of 435 bp. However, in the *O. sativa* spp. *indica* genome ASM465v1, the expected PCR product of marker C365-1 was 418bp (from position of 7,662,135 to 7,662,552). Actually, the PCR product of C365-1 in sterile plants (E15S, TUS) had a band of 435bp (typical of *O. sativa* ssp. *japonica* products) (**Figure 2**) while its products in fertile plants (N97, NTL) showed a band equivalent to 418bp, typical of *O. sativa* ssp. *indica* products (**Figure 2**). The typical PCR products of the C365-1 marker in this study were very similar to the expected product sizes, which were identified by complementing marker primers to the reference genomes (IRGSP-1.0 and ASM465-1.0). However, they were different from the results published by Wang *et al*. (2003), in which the PCR products were approximately 500bp in fertile plants and roughly 520bp in sterile plants. The difference in the genetic background of the breeding materials between ours and those of Wang *et al*. (2013) could be the reason for the difference. Additionally, the PCR products of sterile plants (both E15S and TUS) also included an unexpected product of approximately 370bp. This 370-bp band was very faded compared to the 435-bp band; thus, it seemed to be an atypical product in sterile plants, compared to the clear band of 435 bp (**Figure 2**). Among the 39 waxy plants, MAS indicated that 10 of them were *tms5* homozygous, 19 plants were heterozygous, and 10 plants were *TMS5* homozygous. The genotypic ratio (10:19:10) perfectly fitted the expected ratio of 1:2:1 for a locus with two alleles ($\chi^2 = 0.03$, $P > \chi^2 = 0.99$) (**Table 3**). The results showed that locus C365-1, likely including *tms5*, independently assorted from the locus waxy, as well as the genetic regulators related to important morphological traits.

Finally, there were 10 waxy-TGMS homozygous plants with outstanding agronomic traits selected from a total of 3350 F_2 individuals belong three segregating populations. None of the 10 selected plants were derived from F_2 (E15S × NTL), while five were from F_2 (E15S × N97) and the other five from F_2 (TUS × N97). The cross E15S × NTL did not create any promising recombinants for selection, according to the ideotype, as the two crosses TUS x N97 and E15S × N97. The F_3 seeds, harvested from the 10 plants were F_3 populations for further selection of waxy-TGMS maternal lines.

From the F_3 generation, the selection strategies were similar to those of other TGMS rice developing programs. Remarkably, although selected waxy-TGMS individuals had low-amylose-content grains, this value still varied among segregants because of gene interaction for grain amylose content. Selection on amylose content should be carried out during further selection for better control.

The selection strategies used in this study were very cost-effective for its aims. The morphological selection was carried out first to reduce the number of candidates before further analysis. Segregation analysis for the waxy locus used inexpensive chemical iodine and infrastructure requirements, but still maintained highly reliable results. The MAS for *tms5* required the highest cost in selection, but it was performed last in the selection process so that it could be carried out on a reduced number of candidates after the earlier selection stages.

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