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Full Paper

Breeding of Hawm Gra Dung Ngah rice for photoperiod insensitivity by molecular-marker-assisted backcrossing

Suttirak Plonjarean and Manlika Jindasing *

Department of Crop Production Technology, Maejo University at Chumphon, Lamae, Chumphon 86170, Thailand

* Corresponding author, e-mail: jindasing@hotmail.com

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Abstract: The duration of light during the day or photo-period has a strong influence on the flowering of the rice plant. The purpose of this study is to breed Hawm Gra Dung Ngah (HGDN) rice variety for non-photosensitivity, which will allow farmers to plant it throughout the year and result in increasing rice yields and reduced risks. By backcrossing, molecular markers were used with HGDN rice as the receptor variety and japonica rice variety (RD 55) as the donor parent and the non-photophobic cultivar. It was found that the *hd1* photosensitive genes could be identified by using marker RM 19776 in the seedling stage of the F_1, BC_1F_1, BC_2F_1 and BC_2F_2 populations. The phenotype and genotype ratios of the BC_2F_2 and BC_3F_2 populations conform to Mendel's theory, in which recessive genes are controlled by one gene. The genetic ratio of photoperiod-sensitive rice to non-photoperiod sensitive rice is 3:1, and 26 plants of the BC_2F_3 population have a mean flowering date of 124 ± 3.61 days after planting, cf. 140 ± 3.67 days for the original HGDN rice.

Keywords: Hawm Gra Dung Ngah rice, photoperiod insensitivity, molecular-marker-assisted backcrossing

INTRODUCTION

The flowering period of rice is an agronomic trait that is controlled by genetic and environmental factors [1]. The duration of light during the day or photo-period has an effect on flowering on many crops such as rice. Rice cultivars can be classified according to the length of the day that affects their flowering as photosensitive rice and photo-insensitive rice. The former grows well only in the rainy season. When planted in the dry season with a photoperiod of more than 12 hr/day, it will flower slowly, unlike the photo-insensitive rice that can grow in all seasons of the year [1]. The *Hd1* allele, a light-sensitive allele works over a long period of time by slowing down

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flowering. However, the *hd1* allele, a recessive allele that is not sensitive to light acts to control flowering without responding to the light period and works for a short period of time by promoting inflorescence. Rice therefore is an important cereal crop grown in diverse photoperiods around the world [2]. Although there are many advanced researches on photoperiodism and flowering response in rice, little seems to be known on the genetic complexity of this trait [3], especially the genetic diversity and the genes that regulate this response. Since flowering control is regulated by a large number of genes [4], several photoperiod response genes have been reported to affect yield traits, such as *Ghd7*, *DTH8*/*Ghd8*, *Hd1*, *OsPRR37*/*Ghd7.1*/*DTH7*/*Hd2 RFT1* and *OsMADS51* [5].

In selecting complex traits rice breeders have used the technique of backcrossing with molecular markers, which is efficient, accurate and can save time [2]. Quantitative trait locus (QTL) associated with sensitivity to the *Hd1-Hd14* optical range can be identified by simple sequence repeat (SSR) markers including RM5963, RM8225, RM8226, RM8250, RM19772, RM19776, RM20069 and RM20069 [6].

Hawm Gra Dung Ngah (HGDN) rice is a local rice variety that has been commonly grown for a long time in Narathiwat province in the southern region of Thailand. The highlights of this rice are its ylang-ylang aroma and high nutritional value. However, it is a rice cultivar that is sensitive to photoperiod [7]. Developing a non-photosensitive HGDN rice variety can allow its production all through the year. By selective breeding using molecular markers associated with the *hd1* gene, non-photosensitised rice phenotypes can be screened. Photoinsensitive genes can be transferred to photo-sensitive varieties through backcrossing [3]. The objective of this study is to improve HGDN rice varieties so that they are not photosensitive and can be planted all year round by SSR marker-assisted selection.

MATERIALS AND METHODS

Rice Cultivars

HGDN rice cultivars were selected according to native rice breeding procedure conducted by the Pattani Rice Research Center where rice varieties were collected from farmers' plots in Tak Bai district, Narathiwat province in 2009. By planting and selecting 200 pure cultivars per row, the 59th row yielded the best cultivars with the required characteristic. The strain code PTNC09002-59 was named HGDN rice and certified by the Rice Department.

Plant Breeding Programme

The photoperiod-sensitive rice variety HGDN (recipient parent) was crossed with a photoperiod-insensitive japonica rice variety RD55 (donor parent). The resulting F_1 plants were then backcrossed with HGDN to produce BC_1F_1 seeds. The BC_1F_1 plants were backcrossed with HGDN to produce BC_2F_1 seeds and BC_2F_1 plants were self-pollinated to produce BC_2F_2 seeds. The F_1 plants through to the BC_2F_2 generation were grown in the greenhouse. Molecular-marker-assisted backcrossing technique was used to select the photoperiod-insensitive gene in RD55, F_1, BC_1F_1, BC_2F_1 and BC_2F_2 plants. One hundred and twenty BC_2F_2 plants were exposed to 14 hr of light (1000 lux) everyday to confirm and select photoperiod-insensitivity. BC_2F_3 seeds were harvested from the selected BC_2F_2 plants. Then the BC_2F_3 seeds were planted in 55 single rows (6 m long, 30-cm row spacing). About 120 seeds of each BC_2F_2 plant were grown in a row. Also, HGDN seeds were planted in 5 single rows. The breeding diagram for selecting photoperiod-insensitive BC_2F_2 is shown in Figure 1.

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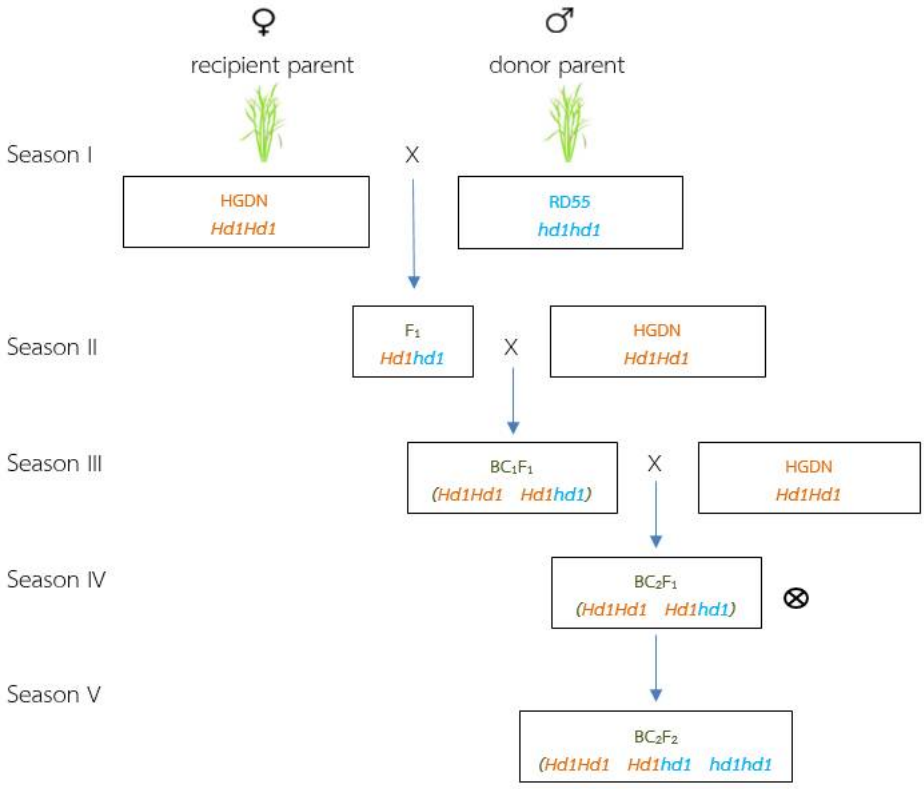

Figure 1. Breeding diagram of the photoperiod-insensitive BC_2F_2

DNA Isolation

Collected leaves of RD55, HGDN, F_1, BC_1F_1, BC_2F_1 and BC_2F_2 plants in plastic bags were placed in ice and later stored at -20 °C. Doyle and Doyle DNA extraction [8] was performed using cetyltrimethylammonium bromide (CTAB) extraction protocol. Briefly, 0.25 g of fresh leaf tissues was ground to powder with liquid nitrogen, then transferred to 1.5 mL microtubes containing 650 µL of 2% CTAB extraction buffer (20 mM EDTA, 0.1 M Tris-HCl, 1.4 M NaCl, 2% CTAB, plus 1% β-mercaptoethanol added just before use). The microtubes were then vortexed and incubated at 60ºC for 45 min. Afterwards, a chloroform extraction was done with 24:1 chloroform: isoamyl alcohol, followed by isopropanol (700 µL) precipitation. DNA pellets were washed twice with 70% ethanol and finally resuspended in 50 µL deionised water. DNAs were electrophoresised on 0.8% agarose gel to verify both the quality and concentration. Fermentas Gene Ruler DNA Ladder Mix (Thermo Scientific, UK) was used as standard for size and concentration.

Marker Analysis

The primers were selected from the quantitative trait locus associated with sensitivity to the *Hd1-Hd14*. SSR analysis of genomic DNA was carried out using RM8225, RM8250, RM19727, RM19776 and RM20069 markers [6]. The primers are listed in Table 1.

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Table 1. List of primers used in SSR analysis

Order	Primer	5/ sequence/3/ sequence	Expected size (bp)
1	RM8225	ATGCGTGTTCAGAAATTAGG TTGTTGTATACCTCATCGACAG	221
2	RM8250	AACCTAAAGGGCAGTTTCC GCGATAAGTTTCTTGTTGATG	171
3	RM19727	ACTGACCTACTCCTCTGCACATTCC GTTTGATCGCGTGTTTGTTGG	184
4	RM19776	ACCTGCTCCATCCATCTCTACGG AGCAACGTGGTACAGATTACAGAAGC	191
5	RM20069	CGAATTCGGCACGAGTAATAGGG GCGAGCGAGAGGAGAGATAGACG	157

SSR Analysis

The polymerase chain reactions (PCR) were carried out for 35 cycles. For the initial denaturation, the PCR were carried out at 95°C for 5 min. The conditions for each cycle were as follows: denaturation at 95°C for 30 sec., annealing at 55-60°C (depending on the annealing temperature of each primer) for 45 sec. and extension at 72°C for 30 sec. The final extension was carried out at 72°C for 5 min. The PCR were done with 2 µL PCR buffer, 0.8 µL 2mM $MgCl_2$, 4 µL 1mM dNTPs, 2 µL DNA template (25 ng), 9.4 µL deionised water, 1.6 µL of each primer (10 pmoles) and 5 units (0.2 µL) of *Taq* polymerase (GenScript, USA). The PCR were performed in a Biometra T-Gradient thermocycler (Biotron, Germany). Samples were mixed with loading-dye buffer and loaded on a 3% agarose gel using Tris-borate EDTA buffer and vertical gel apparatus. The amplification products were size-analysed by electrophoresis for 50 min. at a constant current of approximately 55-65 mA and visualised by ethidium bromide staining. The stained gel was exposed to UV light (260 nm) for 30 min. and kept in the dark. Later, the gels were documented using UVIpro Platinum gel documentation unit (Eppendorf, UK). Bands obtained from parents, F_1 hybrids and BC_2F_2 plants were used to identify photoperiod insensitivity- or sensitivity-inducing alleles at each primer locus.

Primers used for the different assays were chosen on the basis of availability alone and with no previous screening of this plant material. Due to cost considerations and the relatively large amount of DNA required, the SSR analyses were only repeated with the eighteen cultivars. The per cent reproducibility was determined by dividing the number of reproducible bands by the total number of bands observed. The final data sets included both polymorphic and monomorphic fragments. The bands appearing without ambiguity were scored as 1 (present) and 0 (absent) for each primer.

Data Collection

Recorded from ten selected BC_2F_3 plants and ten plants of HGDN were ten agro-morphological characters, namely plant height, days to flowering, days to maturity, number of tillers per hill, number of panicles, seed length, seed width, seed thickness, weight of one thousand grains and yield per plant.

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Statistical Analysis

All data obtained from the HGDN variety and BC_2F_3 population were evaluated using analysis of variance (ANOVA), R program 2.10.10. The traits in HGDN and BC_2F_3 population are reported as means with standard deviations [9].

RESULTS AND DISCUSSION

In marker-assisted backcross breeding, the primer survey is desired for effective foreground, recombinant and background selection. Polymorphic markers are basic for this breeding method. A marker showing dimorphic bands is very essential in the selection, because this marker can separate the two parental genotypes, viz. RD55, the donor parent, and HGDN, the recipient or recurrent. The markers showed very clear recognisable bands that were used for identifying the genetic constitution of the photoperiod-insensitive QTLs very efficiently via electrophoresis. The markers RM8225, RM8250, RM19727, RM19776 and RM20069 were used for parental polymorphism. Only marker RM19776 produced polymorphic bands between photoperiod-insensitive RD55 and photoperiod-sensitive HGDN as shown in Figure 2. Thus, the marker RM19776 was used to identify the photoperiod-insensitive gene (*hd1*). The three F_1 plants were confirmed as true F_1 using the SSR marker RM19776.

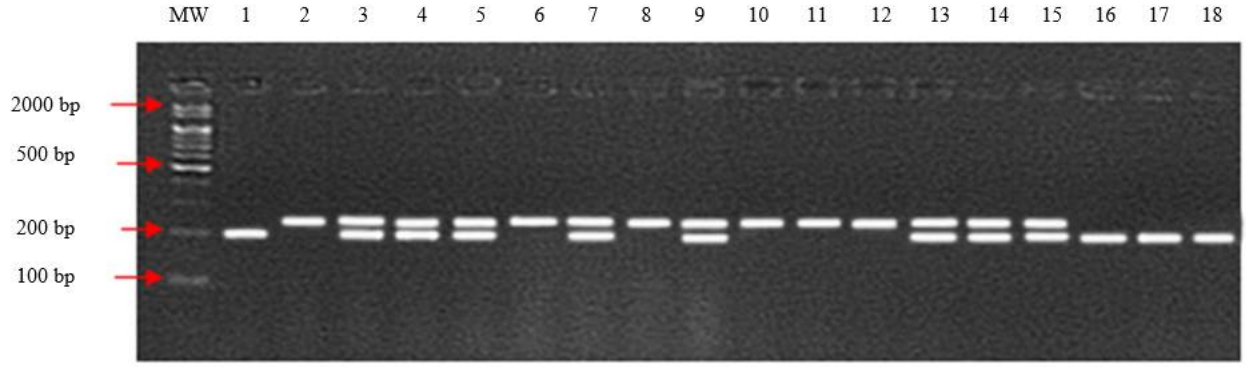

Figure 2. Sample gel photograph under UV light (260 nm) showing DNA banding size from PCR products when using RM 19776 as primer and DNA of different rice varieties as DNA templates: Molecular-weight labelled DNA size 100-2,000 base pair (bp) ladder marker (MW); RD55 (*hd1 hd1*) (lane 1); HGDN (*Hd1 Hd1*) (lane 2); F_1 (*Hd1 hd1*) (lanes 3-5); BC_1F_1 (*Hd1 Hd1 and Hd1 hd1*) (lanes 6-7); BC_2F_1 (*Hd1 Hd1 and Hd1 hd1*) (lanes 8-9); BC_2F_2 (*Hd1 Hd1*) (lanes 10-12); BC_2F_2 (*Hd1 hd1*) (lanes 13-15); and BC_2F_2 (*hd1 hd1*) (lanes 16-18)

As shown in Figure 2, F_1 confirmation and selection were performed on BC_1F_1, BC_2F_1 and BC_2F_2 plants using the tightly linked photoperiod-insensitivity marker RM19776. The DNA bands produced by PCR of photoperiod-insensitive RD55 and photoperiod-sensitive HGDN in some BC_2F_2 plants are also shown. The results are similar to the study of Alam et al. [10], in which foreground selection was done for introgression of *saltol* QTL in rice genotype by marker-assisted backcrossing, which showed that only 33 plants produced flowers which were non-photoperiod sensitive. When tested for their genotypes using marker RM 19776 that was linked with *Hd1/hd1*, all plants that flowered under long day length condition were found to have only one type of genotype, viz. *hd1hd1*.

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Table 2 shows the phenotypic ratios for BC_2F_2 and BC_2F_3 that follow the First Law of the Mendelian Theory for a character controlled by one gene, as segregation ratio for photoperiod: non-photoperiod sensitivity in the ratio of 3:1. Two BC_1F_1 plants and three BC_2F_1 plants carried a photoperiod-insensitive gene so these segregants were selected. As a result of selected photoperiod insensitivity, 33 BC_2F_2 plants bore flowers and 87 BC_2F_2 plants bore no flowers. The BC_2F_2 plants were allowed to grow on a long daylength period of 14 hr per day.

Table 2. Phenotypic ratios of photoperiod sensitivity/non-photoperiod sensitivity using chi-square test (χ^2)

HGDN Population	Observed phenotypic value		Test of phenotypic ratio	
	Photoperiod sensitive (non-flowering)	Non-photoperiod sensitive (flowering)	Expected ratio (photoperiod : non-photoperiod sensitivity)	Chi-square χ^2
BC_2F_2	87	33	3:1	3.09*
BC_2F_3	94	26	3:1	3.21*

* With statistically significant difference when compared with tabular chi-square value of 3.84 at 95% confidence level

Agronomic traits and morphology of HGDN and BC_2F_3 populations are shown in Table 3. The means of days to flowering, days to maturity, one-thousand-grain weight and grain yield are lower in the BC_2F_3 population than the HGDN population. Linkage drag or undesirable genes are often found during marker-assisted backcrossing [10]. Rice breeders can use flanking markers to reduce possible linkage drag and about 99% of recurrent parent genome can be recovered after four backcross generations (BC_4) in marker-assisted backcrossing [12]. Feng et al. [13] have reported 99.89% recurrent parent genome recovery only after the third backcrossed generation in rice. The mean of plant height, seed length, seed width and seed thickness are also lower or in lower trend in the BC_2F_3 population than HGDN (Table 3, Figure 3). These results indicate that the chromosome fragments derived from RD55 can affect the yield, yield component and other traits. BC_2F_3 rice plant flowered at an average of 117-126 days after planting, cf. 136-145 days for the starting HGDN rice.

On the other hand, as shown in Table 3 and Figure 4, the mean of number of tillers in the BC_2F_3 population (10 ± 2.53) is higher than the recurrent parent with 8 ± 0.58 numbers of tillers. The mean number of panicles in the BC_2F_3 population,10 ± 2.31, is also higher than the recurrent parent with 8 ± 0.81. These results are similar to those by Watcharin et al. [14].

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Table 3. Morphology and agronomic traits of HGDN and BC_2F_3 populations, the latter being generated using HGDN as recurrent parent and RD55 as donor parent

Trait	HGDN	BC_2F_3		P-value
	Mean ± SD	Mean ± SD	Range	
Plant height (cm)	166 ± 9.31	149 ± 4.18	139 – 161	**
Days to flowering	140 ± 3.67	124 ± 3.61	117 – 126	*
Days to maturity	173 ± 2.42	137 ± 4.26	132 – 143	*
Number of tillers	8 ± 0.58	10 ± 2.53	6 – 12	*
Number of panicles	8 ± 0.81	10 ± 2.31	7 – 11	*
Seed length (mm)	8.97 ± 0.64	7.78 ± 2.37	7.05 – 8.98	*
Seed width (mm)	2.22 ± 0.10	2.18 ± 0.35	2.02 – 2.34	*
Seed thickness (mm)	2.32 ± 0.12	2.11 ± 0.13	2.06 – 2.83	*
One-thousand-grain weight (g)	23.03 ± 1.53	21.45± 2.12	18.13 – 24.21	ns
Grain yield (g/plant)	19.43 ± 4.26	16.81 ± 2.62	15.14 – 22.38	ns

** highly significant, * significant, ns = non-significant

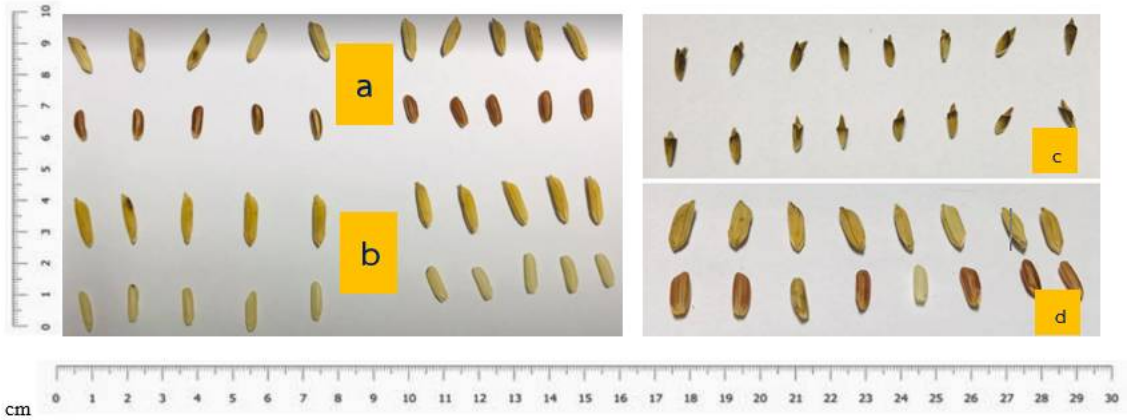

Figure 3. Seeds of a) HGDN; b) RD55; c) F_1 population; d) BC_2F_3

Figure 4. Tiller and panicle numbers: a) HGDN; b) BC_2F_3

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CONCLUSIONS

A major gene, *Hd1/hd1*, of BC_2F_2 has been derived from genetic improvement of HGDN rice variety. The marker RM19776 was used to identify true hybrids and the presence of a photoperiod-insensitive gene (*hd1*). Flowering time is one of the important aspects which determine regional and seasonal adaptation and has been a main target of selection in rice breeding programmes. BC_2F_3 rice was found to have a flowering time of 117-126 days after planting and is photoperiod-insensitive. In the next process test planting of the rice cultivars obtained will be carried out in different environments at the cultivar testing station.

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