
Improving photoperiod insensitivity of a Thai upland rice variety by marker-assisted foreground selection

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Abstract Global warming might increase climate variability with abnormal rainfall distributions. Photoperiod insensitive upland rice can be grown early or late in the rainy season, and to help the stabilize upland rice yields and reduce the risks to farmers. The SSR marker-assisted backcrossing method was applied to breed photoperiod insensitivity into the Thai superior upland rice variety cv. Dawk Pa-yawm as the recurrent parent. The donor parent was photoperiod insensitive Taichung 65, a japonica variety. The photoperiod insensitivity gene *hd1* was identified using the RM8225 marker in seedling stage of F₁, BC₁F₁, BC₂F₁ and BC₂F₂ plants. The photoperiod insensitive BC₂F₂ plants that flowered were selected during long day length. The results demonstrated successful use of genetic markers to alter the photoperiod insensitivity trait of an elite rice variety.

Keywords: DNA marker, Backcross breeding, *Oryza sativa* L., SSR

Introduction

Plant breeders have used conventional backcrossing to transfer targeted genes from a donor into an elite cultivar. The target gene was selected by the phenotype it expresses (Allard, 1960). DNA markers, such as simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs), have been used to identify and select target genes of donors in marker-assisted backcrossing (MABC) (Hasan *et al.*, 2015). Plant breeders can directly select a gene at the seedling stage or from a single selected plant. In the first reported application to rice of MABC, the bacterial blight (BB) resistance gene was transferred into a restorer line, Minghui 63 (Chen *et al.*, 2000). MABC technique has been used to improve several rice traits of elite varieties, such as BB resistance, blast resistance, submergence tolerance, salt tolerance, drought tolerance, aroma, and

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photoperiod insensitivity (Chen *et al.*, 2000; Collard and Mackill, 2007; Hasan *et al.*, 2015).

In southern Thailand, the upland rice (*Oryza sativa* L.) varieties grown in the rainy season are short-duration photoperiod-sensitive varieties, such as Dawk Pa-yawm. However, global warming might induce abnormal rainfall distributions. If rice breeders could provide photoperiod insensitive upland rice varieties, these could be grown early or late in the rainy season, improving stability of upland rice production.

The photoperiodic control of flowering is regulated by many genes. Fourteen leading related QTLs of rice, *Hd1-Hd14*, were identified by Lin *et al.* (2000); Lin *et al.* (2002); Yamamoto *et al.* (1998); Yamamoto *et al.* (2000) and Yano *et al.* (2001). SSR markers, such as RM5963, RM8225, RM8226 and RM8250 tightly linked with a photoperiod insensitivity gene (*hd1*) of Taichung 65 variety, were reported by Sangtong *et al.* (2007) and Sangtong *et al.* (2008). Thus, the photoperiod insensitivity gene could be transferred to photoperiod sensitive varieties by using marker-assisted backcrossing to select for the photoperiod insensitivity gene.

The specific objective of this study was to improve the superior upland rice variety of southern of Thailand cv. Dawk Pa-yawm, by giving it photoperiod insensitivity through SSR marker-assisted backcrossing.

Materials and methods

Parents and Development of BC₂F₃

The scheme for selecting photoperiod insensitive BC₂F₂ is shown in Figure 1. An indica upland rice photoperiod sensitive variety, Dawk Pa-yawm (♂), was crossed with a japonica photoperiod insensitive variety, Taichung 65 (♀). Then the resulting F₁ plants were backcrossed to Dawk Pa-yawm to produce BC₁F₁ seeds. The BC₁F₁ plants were backcrossed to Dawk Pa-yawm to produce BC₂F₁ seeds. The F₁ to BC₂F₂ generation advance was produced in a greenhouse. The MABC technique was used to select for photoperiod insensitivity gene in Taichung 65, in F₁, BC₁F₁, BC₂F₁ and BC₂F₂ plants. The 200 BC₂F₂ plants were exposed to 15 hours of light (1000 lux) every day to confirm and select for photoperiod insensitivity. The BC₂F₃ seeds were harvested from the selected BC₂F₂ plants. Then the BC₂F₃ seeds were planted into 53 single rows (5 m long; 30 cm row spacing). About 50 seeds of each BC₂F₂ plant were planted in a row. Also Dawk Pa-yawm seeds were planted into 4 single rows.

Sample collection and DNA extraction

Young leaves of Taichung 65, Dawk Pa-yawm, F₁, BC₁F₁, BC₂F₁ and BC₂F₂ plants were collected and put into plastic bags placed in ice, and were later frozen and stored at -20 °C. DNA from rice leaf tissue was extracted with N-Cetyl-N,N,N-trimethylammonium bromide method (CTAB) of Dellporta *et al.* (1983) with slight modifications. Leaf tissue of 0.2 g was ground to fine powder in the presence of liquid nitrogen using mortar and pestle. The powder was then transferred into a 1.5 ml Oak Ridge tube, mixed with 700 µl extraction CTAB buffer (PVP-40 1%, 1.4 mM NaCl, 20 mM Na₂EDTA pH 8.0, and CTAB 2%) as well as 2% β-Mercaptoethanol, and incubated at 65 °C for 1 hour. Then 700 µl of Chloroform:Isoamyl alcohol (24:1 v/v) was added with shaking for 20 mins. The mixture was centrifuged at 12,000 rpm for 15 mins at 4 °C to separate leaf residues. The supernatant was collected into a new tube and was mixed with 700 µl isopropanol to precipitate the DNA. After discarding the supernatant, the DNA pellet was washed with 70% ethyl alcohol (500 µl) two times for 5 mins each time, dried, and then 30 µl of TE buffer was added. DNA concentration and purity of these samples were measured using a spectrophotometer at wavelengths 260 and 280 nm.

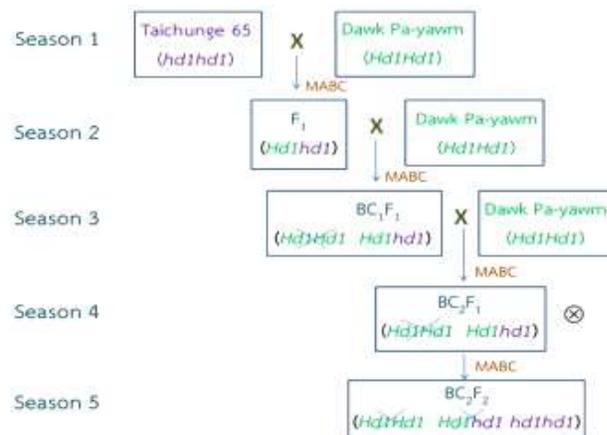


Figure 1. Development of the photoperiod insensitive BC₂F₂

SSR analysis and primer screening

SSR analysis of genomic DNA was carried out using RM5963, RM8225, RM8226 and RM8250 markers located at the distances 0.6, 0, 0 and 7.5 cM from the *hd1* gene, respectively (Sangtong *et al.*, 2007; Sangtong *et al.*, 2008). The primers are listed in Table 1. The PCR reaction blend had 20 ng of

genomic DNA, 2 μ l of 10X Taq buffer, 0.2 μ M of each primer in forward and reverse primer pair, 200 μ M of each of the four dNTPs, and 0.7 units of Taq polymerase. PCR amplifications were carried out on a Biometra thermo cycler using the following program: denaturation at 94 °C for 3 mins 1 cycle, annealing at 52 or 55 °C (depending on the primer used) for 1 min, extension at 72 °C for 1 min for 30 cycles, and a final elongation step at 72 °C for 5 mins. The products were stored at -20 °C. The PCR products were later separated by gel electrophoresis using 3% (W/V) agarose gel, run at a constant 100 V for 60 mins. The gel was stained with ethidium bromide for 15 mins and de-stained with double-distilled water for 15 mins. The bands were detected under UV lights. Bands of parents, F₁ hybrids and BC₂F₂s were used to identify photoperiod insensitivity or sensitivity inducing alleles at each primer locus.

Table 1. List of the primers used in SSR analysis

Order	Primer	Position on Chromosome 6 (cM)	5' sequence/3' sequence	Expected size (bp)
1	RM5963	53.5	CTGCCTAGCTTCCGTTTCTC AGTTACGGGAAATGTGTGGC	196
2	RM8225	54.1	ATGCGTGTTCAGAAATTAGG TTGTTGTATACCTCATCGACAG	221
3	RM8226	54.1	TTAGGATACGGCTTCTAGGC CGTAATTGTTGCATATGGTG	251
4	RM8250	61.6	AACCTAAAGGGCAGTTTCC GCGATAAGTTTCTTGTGATG	171

Source: McCouch *et al.* (2002)

Data collection

Nine agro-morphological characters were recorded on five selected BC₂F₃ plants and ten plants of Dawk Pa-yawm, namely plant height (cm), days to flowering (day), days to maturity (day), number of tillers per hill, number of panicles, seed length (cm), seed width (cm), weight of one thousand grains (g) and yield per plant (g).

Statistical analysis

All obtained data from the Dawk Pa-yawm variety and the BC₂F₃ population were evaluated to analysis of variance (ANOVA) using R programe with agricolae package (Mendiburu and Simon, 2007). The traits in Dawk Pa-yawm and BC₂F₃ population are reported as means with standard deviations (Dowdy *et al.*, 2003).

Results

In primer survey the markers RM5963, RM8225, RM8226 and RM8250 were used for parental polymorphism. Only marker RM8225 produced polymorphic bands between photoperiod insensitive Taichung 65 and photoperiod sensitive Dawk Pa-yawm (Figure 2). Thus, the marker RM8225 was used to identify a photoperiod insensitive gene (*hd1*). The 5 F₁ plants were confirmed as true F₁ using the SSR marker RM8225. Figure 2 shows the gel picture of F₁ confirmation. All the F₁s were true hybrids.

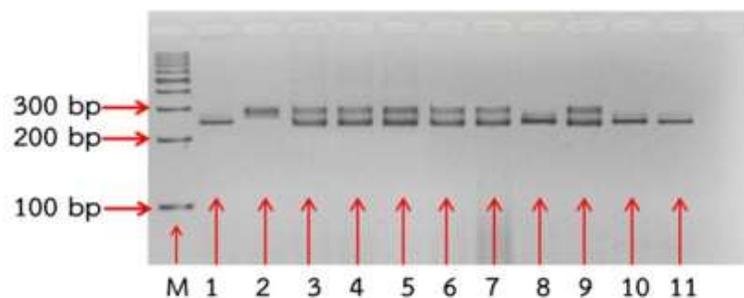


Figure 2. Example of PCR products using RM8225; M labels the DNA size ladder marker; lane 1 is for Taichung 65 (*hd1hd1*); lane 2 for Dawk Pa-yawm (*Hd1Hd1*); lanes 3-7 for F₁ (*Hd1hd1*); lanes 8 and 10-11 for BC₂F₂ (*hd1hd1*); and lane 9 for BC₂F₂ (*Hd1hd1*)

Foreground selection was performed on BC₁F₁, BC₂F₁ and BC₂F₂ plants using the tightly linked photoperiod insensitivity marker RM8225. Figure 2 shows the DNA bands produced by PCR of photoperiod insensitive Taichung 65 or photoperiod sensitive Dawk Pa-yawm in some BC₂F₂ plants.

The results show that two BC₁F₁ and three BC₂F₁ plants carry a photoperiod insensitivity gene, so these segregants were selected. As a result of selected photoperiod insensitivity, 53 BC₂F₂ plants had flowers and 147 BC₂F₂ plants had no flowers.

The morphology and agronomic traits of Dawk Pa-yawm and BC₂F₃ populations are shown in Figure 3 and Table 2. The means of plant height (63 cm), seed length (5.22 cm) and seed width (2.52 cm) were lower in the BC₂F₃ population than in Dawk Pa-yawm with plant height (112 cm), seed length (9.67 cm) and seed width (2.16 cm). The means of days to flowering (93 days), days to maturity (123 days), one thousand grain weight (19.76 g) and grain yield (7.75 g/plant) were trending lower in the BC₂F₃ population than in Dawk Pa-yawm with days to flowering (96 days), days to maturity (125 days), one thousand grain weight (21.42 g) and grain yield (10.50 g/plant).

Table 2. Yield and other characteristics of Dawk Pa-yawm and BC₂F₃ populations, the latter generated using Dawk Pa-yawm as the recurrent parent and Taichung 65 as the donor parent

Trait	Dawk Pa-yawm	BC ₂ F ₃		F-test	CV(%)
	Mean ±SD	Mean ±SD	Range		
Plant height (cm)	112 ±12.61	63 ±3.97	56 - 68	**	11.9
Days to flowering (day)	96 ±1.47	93 ±2.94	88 - 97	ns	2.7
Days to maturity (day)	125 ±1.47	123 ±2.94	118 - 127	ns	2.1
Number of tillers (no.)	4 ±0.8	5 ±1.41	3 - 7	ns	27.3
Number of panicles (no.)	3 ±0.63	5 ±1.47	3 - 7	*	30.8
Seed length (cm)	9.67 ±0.53	5.22 ±0.32	4.77 - 5.93	**	6.3
Seed width (cm)	2.16 ±0.10	2.52 ±0.18	2.24 - 2.83	**	6.6
One thousand grain weight (g)	21.42 ±1.82	19.76 ±1.26	18.3 - 21.1	ns	8.5
Grain yield (g/plant)	10.50 ±4.77	7.75 ±1.79	6.12 - 10.09	ns	44.2

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

The mean of number of panicles (5 no.) in the BC₂F₃ population were higher than in the recurrent parent with number of panicles (3 no.). The mean of number of tillers (5 no.) in the BC₂F₃ population were trending higher than in the recurrent parent with number of tillers (4 no.).



Figure 3. a: Plant type of Dawk Pa-yawm (left) and BC₂F₃ population (right); scale bar represents 6 cm. **b:** Seeds of Dawk Pa-yawm (left) and BC₂F₃ population (right); scale bar represents 0.5 cm

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. Taichung 65, the donor parent, and Dawk Pa-yawm, the recipient or recurrent. The marker showed very clear bands. It was available to identify the genetic constitution of the photoperiod insensitive QTLs very

efficiently via agarose gel electrophoresis. Thus, the marker RM8225 was used to identify true hybrids and presence of a photoperiod insensitive gene (*hd1*). The results were similar as in the study of Alam *et al.* (2012), in which foreground selection was done for introgression of *saltol* QTL into rice genotype by marker-assisted backcrossing.

The means of plant height, days to flowering, days to maturity, seed length, seed width, one thousand grain weight, and grain yield were lower or trending lower in the BC₂F₃ population than in Dawk Pa-yawm. These results indicate that the chromosome fragments derived from Taichung 65 could affect the yield, yield component, or other traits. Lewise *et al.* (2007) showed that linkage drag or undesirable genes are often found during marker-assisted backcrossing. Rice breeders can use flanking markers to reduce possible linkage drag. About 99% of recurrent parent genome can be recovered after four backcross generations (BC₄) in marker-assisted backcrossing (Hasan *et al.*, 2015). Feng *et al.* (2017) have reported 99.89% recurrent parent genome recovery after only the third backcross generation in rice.

Breeding of the superior upland rice cv. Dawk Pa-yawm for photoperiod insensitivity was demonstrated by using SSR marker-assisted backcrossing in seedling stage, and the SSR marker used was RM8225. In future studies, developed photoperiod insensitive lines need to use background selection to obtain other Dawk Pa-yawm genomes.

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