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PHENOTYPIC AND GENOTYPIC ANALYSIS FOR EARLY MORNING FLOWERING TRAITS AT FLOWERING STAGE IN RICE (*Oryza sativa* L.)

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Abstract

The study focused on phenotypic and genetic factors underlying early morning flowering traits in rice in the hope that rice genetic improvement in the warming future will be made possible. Quantitative trait loci (QTL linked to early morning flowering (EMF) traits was analyzed by selective genotyping of phenotypic extremes among 684 BC_1F_2 lines (derived from the cross PSB Rc82 x WAB56-125) under glasshouse condition was undertaken using single marker analysis (SMA). Four parameters of flower opening time or start time (FOT), peak of flowering (POF), flower mostly closed (FMC), and flower closed (FC) time were recorded. Results of SMA revealed three markers possibly linked to FOT on chromosome 5, 7, and 9, while 6 markers were possibly linked to POF, FMC and FC time on chromosomes 5, 7, 8, 9, and 11. Generally, markers on chromosomes 5, 7 and 9 indicated QTLs controlling EMF during flowering stage in rice. While this needs to be confirmed using a larger population size and other mapping methodology, the results could already be useful for further fine mapping and eventually for marker-assisted selection of heat escape rice cultivars in the future.

Keywords: Early morning flowering, high temperature, QTL analysis, rice

INTRODUCTION

The advent of climate change brought by global warming is a serious threat in rice production, agricultural productivity, farm incomes, and global food security in general. Global warming is estimated to increase temperature by 0.2 ºC per decade thus it poses a serious threat to rice production (Wassman and Dobermann, 2010; Yang *et al.*, 2007). In China, 3 million hectares of rice were damaged and about 5.18 million tons of paddy rice were lost in 2003 along the Yangtze River Valley due to a heat wave of above 38°C lasting for more than 20 days and coinciding with the flowering stage (Lin *et al*. 2004, Xia and Qi 2004, Yang *et al*. 2004). Likewise, severe yield losses were experienced in 2006 and 2007 in South China (Zou *et al*. 2009) and the Kanto and Tokai regions of Japan during the summer of 2007 (Hasegawa *et al*. 2009). Furthermore, simulations by Horie *et al*. (1996) predicted that the yield of current rice varieties in southern Japan would be reduced by up to 40% in future climates. In Tanzania, a projected seasonal temperature in 2050 increases by 2 °C that could reduce average rice yields by 7.6% (Rowhani *et al*., 2011).

Generally, rice in tropical countries is cultivated at its most favorable day/night temperature of 28/22 °C (Redoña *et al.*, 2007). However, further increase of 10-15°C temperature above optimum is considered heat stress (Wahid *et al.*, 2007). Heat stress happens when plants are exposed to high temperature for a period of time sufficient to cause permanent damage to plant growth and development (Ismail and Hall, 2007). The most sensitive growth stage of rice to heat stress is flowering time (Mackill *et al*., 1982; Kuang *et al*. 2002) and rice seed set is very susceptible to high temperature during flowering (Yoshida 1981). Breeding rice for high temperatures has been attempted only recently but progress has been made to date. There are two approaches in developing or breeding rice varieties adapted to high temperatures: first is to breed rice for heat tolerance *per se* (or true heat tolerance); second is to breed rice that would

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avoid high temperature or heat escape (Redoña *et al.*, 2007). A recent study showed that flower opening in the early morning (heat escape) helps prevent sterility of rice caused by heat stress at flowering and this is under genetic control and affected by weather, particularly air temperature (Kobayasi *et al.*, 2010).

The study aimed to map QTLs for early morning flowering (EMF) in rice and identify markers closely linked to major QTLs. Once fine mapped and cloned, these genes or regions will eventually be used in marker assisted selection to develop EMF varieties and this will accelerate the breeding process in EMF improvement. The detection of SSR markers closest to the QTLs makes easy the study concerning QTL mapping of EMF genes in rice. This study was conducted at International Rice Research Institute from July 2007 to July 2010. It was conducted at the NS02 greenhouse of Plant Breeding, Genetics and Biotechnology Division (PBGBD) and in the Gene Array and Molecular Applications (GAMMA) laboratory of the International Rice Research Institute (IRRI), Laguna, the Philippines (14°30'N, 121°1'E) from November 2007 to July 2010. Early morning flowering evaluation for BC_1F_2 population was done at BG03-b glasshouse.

MATERIALS AND METHODS

Plant Materials and Mapping Population

Two diverse rice varieties – PSB Rc82 (*Oryza sativa*) and WAB56-125 (*Oryza glaberrima*-derived) were used as parents in developing mapping populations in this study. PSB Rc82 is a high yielding rice variety which is susceptible to high temperature stress whereas WAB56-125 is a heat-escape cultivar which manifests some heat tolerance *per se*. Prasad et al. (2006) reported that generally, *Oryza sativa* genotypes will flower between 1000 and 1200 h, whereas *Oryza glaberrima* genotypes completed flowering by 0900 h. Sheehy et al. (2005) proposed that early morning flowering (EMF) trait could be beneficial for reducing yield loss by avoiding or escaping from the damaging effects of high temperature.

The mapping population was developed from a cross between PSB Rc82, a heat-sensitive variety and WAB56-125, a heat-escape donor parent. The F_1 plants derived from this cross (PSB Rc82 x WAB56-125) were subsequently backcrossed to PSB Rc82 (recurrent parent) to generate BC_1F_1. The 235 BC_1F_1 progenies were subjected to heat tolerance screening in internal growth chambers (IGC, Thermoline, Austraia) of IRRI with 6 h of high temperature (38°C) setting each day during flowering time. A temperature setting in the IGC was set to simulate the temperature in the field (Table 1). The same materials were selfed to generate 684 BC_1F_2 progenies that were screened and evaluated for early morning flowering (EMF) trait like flower opening or start time (FOT), peak of flowering (POF), flower mostly closed (FMC), and flower closed (FC) time.

Early Morning Flowering of BC_1F_2 population

Early morning flowering (EMF) traits were evaluated using 685 BC_1F_2 plants and their parents (PSB Rc82 and WAB56-125) under glasshouse condition at IRRI to determine spikelet opening or start time, peak time when 50% of the spikelets are opened, time when most of the spikelets are closed and, lastly, time when all spikelets are closed among the BC_1F_2 plants. To investigate these traits, an intensive data gathering was made by recording every after 30 minutes from 6:00 AM to 1:30 PM the time when the first spikelet opened, the peak time when 50% of the spikelets opened, time when most of the spikelets are closed and, lastly, time when all spikelets are closed. All panicles of each BC_1F_2 individuals were evaluated for these flowering parameters. EMF is a unique trait where the flowers open at cooler and earlier time of the day as an escape mechanism to high temperature-induced sterility during flowering. Kobayashi et al. (2010) reported that flower opening in the early morning helps avoid sterility in rice caused by heat stress at anthesis.

Genotyping of P_1, P_2, and BC_1F_2 Population

Genomic DNA was extracted from 685 BC_1F_2 progenies including the parents using the modified cetyltrimethylammoniumbromide (CTAB) method (Murray and Thompson, 1980). A total of 164 polymorphic SSR markers were identified between parents: PSB Rc82 and WAB56-125. Only 84 SSR markers with distinct banding patterns and having 10-20 centiMorgan (cM) genetic distance evenly distributed across the 12 rice chromosomes were utilized to screen the genotypes of the BC_1F_1

population. Primer sequences were obtained from Gramene database (http://www.gramene.org/). Phenotypic extremes from the best tail (highly fertile) and worst tail (highly sterile) were chosen for selective genotyping, with each tail containing genomic DNA from the two sets of extreme BC_1F_2 progenies. These tails were the top 27 heat tolerant (best tail) and the other 16 heat-sensitive extreme (worst tail) from the whole population. This method saves time, effort, and resources and for these reasons selective genotyping was used in this study. The rationale behind the method is that in a QTL mapping population, some progeny contribute more linkage information than others. The individuals that provide the most linkage information are those genotypes which can clearly be inferred from their extreme phenotypes (Lander and Botstein 1989). PCR and PAGE were carried out rigorously before the stained gels were visualized in the Alphaimager® Gel Documentation device for DNA band scoring. Clear electrophoretic bands generated from each primer were scored as "A" for homozygous sensitive parent, "B" for homozygous tolerant parent and "H" for heterozygote. Since the mapping population used was a backcross-derived population, only two genotypes were observed across the population, homozygous for one parent and heterozygote.

QTL Analysis

The genetic map used was based on the map constructed by the Cornell University developed using the population of doubled-haploid lines (DH) derived from the inter-subspecific cross between IR64 (*indica*) and Azucena (*japonica*) varieties (McCouch *et al*, 1997). The QTLs associated with spikelet fertility, pollen fertility, and Early Morning Flowering traits (flower opening time, peak of flowering, flower mostly closed, and flower closed) were identified using single marker analysis (SMA). SMA is the simplest way for detecting QTLs associated with single markers. Single-marker analysis to detect main effect of QTL was performed by MINITAB 14.0 (Minitab Inc., State College, PA, USA). Significant association of a tested marker with a QTL for heat tolerance was detected by one-way ANOVA. All statistical procedures were performed with MINITAB 14.0 (Minitab Inc., State College, PA, USA).

Marker-Phenotype Association Analysis

SSR primer pairs which generated polymorphic markers between PSB Rc82 and WAB56-125 were used to detect the polymorphic markers associated with the phenotype using the DNA of phenotypic extremes (tails) of BC_1F_2 lines for early morning flowering (EMF) traits. The SSR primer pairs producing polymorphism between parents – PSB Rc82 and WAB56-125 were surveyed on selected (the 16 heat-sensitive and 27 heat-tolerant BC_1F_2 plants) BC_1F_2 progenies to evaluate the segregation of the markers.

RESULTS AND DISCUSSION

Early Morning Flowering (EMF) of the BC_1F_2 population

Rice flowering time usually occurs within 1000H-1200H (Nishiyama and Blanco 1980). Flower opening of rice in early morning is a useful response to avoid heat-induced sterility at anthesis, considering that sensitivity of rice flowers to high temperatures decreases within the 1 h period after flower opening (Satake and Yoshida 1978). Prasad et al. (2006) claimed that rice genotypes can either escape or avoid heat stress at flowering by heading during the cooler periods of the season or by flowering during cooler hours of early morning.

Early morning flowering (EMF) of PSB-Rc82, WAB56-125 and their BC_1F_2 plants were evaluated under glasshouse conditions at IRRI from April 4, 2010 to June 11, 2010.

This was done in the glasshouse to avoid the effect of other environmental factors. Relative time when flowers start to open (FOT), peak flowering time (PFT), time when most of the flowers are closed (FMCT), and time when all of the flowers are closed (FCT) were observed and recorded. Results revealed that WAB56-125 started opening spikelets earlier than PSB-Rc82. Closing of spikelets was also earlier in WAB56-125 than PSB-Rc82 at 0930H and 0945H, respectively. This could be a potential escape mechanism for WAB56-125 in order to avoid the high temperature during the day. On the other hand, variations in FOT, PFT, FMCT, and FCT were observed among the 684 BC_1F_2 plants. Among the BC_1F_2 plants evaluated based on these parameters,

the most stable and the earliest flowering plants were BC_1F_2-227-3, BC_1F_2-229-4 and BC_1F_2-195-1. These materials are promising source for the EMF trait which exhibit escape mechanism under high temperature stress condition during the day. EMF is a unique trait where the flowers open under cooler and earlier time of the day as an escape mechanism to high temperature-induced sterility during flowering. Kobayashi et al. (2010) reported that flower opening in the early morning helps avoid sterility in rice caused by heat stress at anthesis.

Start of Flowering

The normal distribution of time for flowers to start opening of 684 BC_1F_2 progenies derived from the 235 BC_1F_1 plants compared to their parents is shown in Figure 1. Majority of the BC_1F_2 plants started opening spikelets at 480 min (0800H). WAB56-125 opened its spikelets at 431.67 min (0811H), than PSB-Rc82 at 495 minutes (0915H). Likewise, WAB56-125 had earlier spikelet opening compared with most of the 684 BC_1F_2 plants. This could be attributed to the genetic make-up of WAB56-125 having been derived from a cross between *Oryza sativa* L. and *Oryza glaberrima* Steud. Generally, *glaberrima* Steud and its hybrids derived from interspecific crosses to *O. sativa* open flowers earlier than those of *sativa* L. (Nishiyama and Blanco 1980; Jagadish et al. 2008; Nishiyama and Satake 1981). A recent study showed that start of flower opening time in the early morning is an escape mechanism to avoid spikelet sterility caused by heat stress at flowering in rice (Kobayashi et al. 2010).

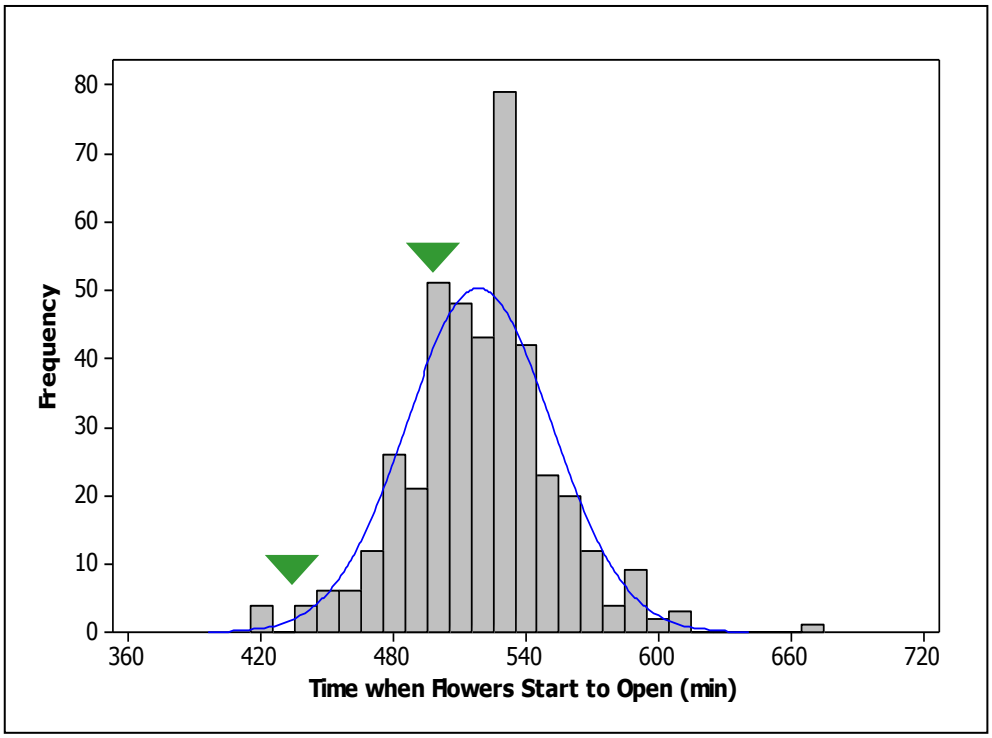

Figure 1. Frequency distribution of the start of flower opening in 684 BC_1F_2 progenies and their parents PSB-Rc82 and WAB56-125.

Peak of Flowering

Peak flowering in this study was considered when 50% of the spikelets were opened (Thanh et al. 2010). Figure 2 illustrates the normal distribution of peak flowering among 684 BC_1F_2 plants and their parents. Since it fit the normal distribution, this indicates that

the time of peak flowering is controlled by multiple genes. The relative values of peak flowering among the 684 BC_1F_2 plants ranged from 465 min (0745H) to 690 min (1130H), with an average of 561.77 min (0922H). WAB56-125 had its peak flowering at 477.5 min (0757H) which was earlier than PSB-Rc82 that peaked at 547.50 min (0907H). In addition, WAB56-125 was relatively earlier to reach the peak of flowering than the majority of the BC_1F_2 population.

Peak of flowering is a very important parameter for the early morning flowering trait since this is the time when most (≈50%) of the spikelets are opened during the day, the peak of pollination is where subsequent events before fertilization occur, and the most sensitive stage in rice to heat stress (Mackill *et al*. 1982; Kuang *et al*. 2002; Yoshida 1981). High temperature that coincides with flowering would result to sterility due to poor anther dehiscence and decreased pollen growth, hence reduced numbers of pollen grains germinating on the stigma (Matsui et al. 2000, 2001; Prasad et al. 2006). Advancing the peak of flowering at early hours in the morning would mean escaping high temperatures during later hours of the day (Prasad et al. 2006). Avoidance of high temperature stress even for 1 hour during flowering is sufficient to reduce spikelet sterility (Jagadish et al. 2007).

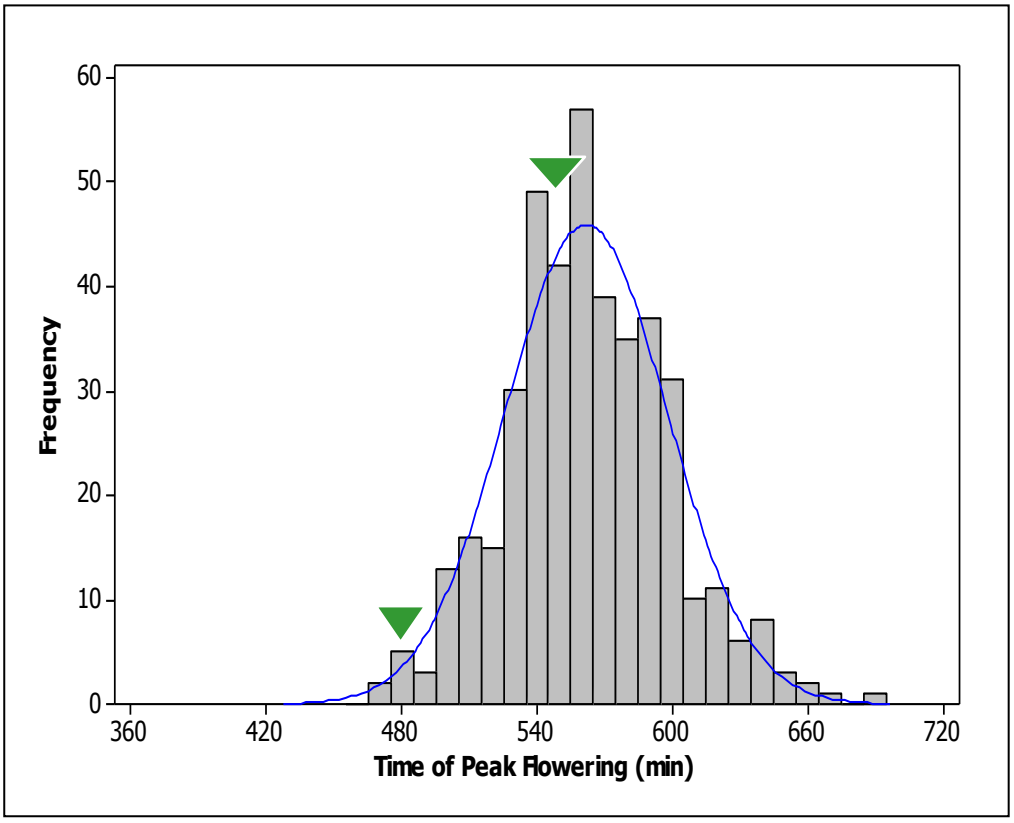

Figure 2. Frequency distribution of the peak of flowering in 684 BC_1F_2 plants and their parents PSB-Rc82 and WAB56-125.

Most of the Flowers are Closed

Figure 3 shows the normal distribution of the time when flowers (spikelets) were mostly closed in 684 BC_1F_2 plants and their parents. For the 684 BC_1F_2 plants, the closing of flowers ranged from 510 min (0830H) to 750 min (1230H) with a mean of 630 min (1030H). WAB56-125 had most of its spikelets closed at 536.67 min (0857H) which was earlier than that of PSB-Rc82 at 585 min (0945H). In addition, WAB56-125 had most of its spikelets closed relatively earlier than the majority of the BC_1F_2 plants.

When most of the spikelets are closed it is considered to be safe from the negative effect of high temperature stress. This is a morphological mechanism of the rice spikelet to protect its pollination and fertilization processes, although no studies have been conducted yet to determine the closing time of spikelets in association with spikelet sterility. Sterility does not occur when spikelets close 1 h before high temperature treatment, suggesting that spikelets have considerably high tolerance after completion of fertilization as claimed by Satake and Yoshida (1978).

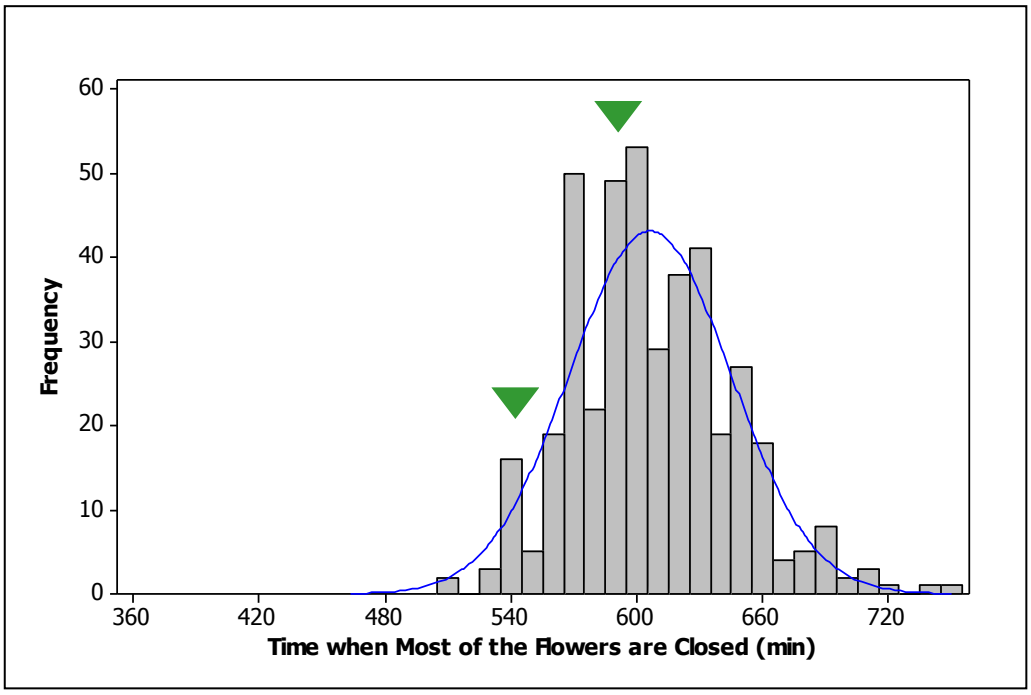

Figure 3. Frequency distribution of the time when most of the flowers are closed in 684 BC_1F_2 plants and their parents PSB-Rc82 and WAB56-125.

Flower Closing Time

Closing of spikelets at cooler temperature (before 0930H or ≤570 min) in the morning means full protection against high temperature-induced sterility in rice, and could be a good index for selecting heat escape materials (Howell 2010, Personal Communication). Figure 4 shows the normal distribution of flower closing time among 684 BC_1F_2 plants and their parents, indicating that flower closing time is controlled by several genes and is fit for QTL analysis. Flower closing time among the 684 BC_1F_2 plants ranged from 540 min (0900H) to 780 min (1300H) with a mean of 638.78 min (1039H). It can be inferred that majority of the BC_1F_2 plants closed spikelets from 1000H to 1100H, with the highest number of plants (≈50) that have spikelets closing at 1000H. WAB56-125 closed earlier at 566.67 min (0927H) than PSB-Rc82 with 600 min (1000H). Consistently, WAB56-125 closed its spikelets relatively earlier than most of the BC_1F_2 plants.

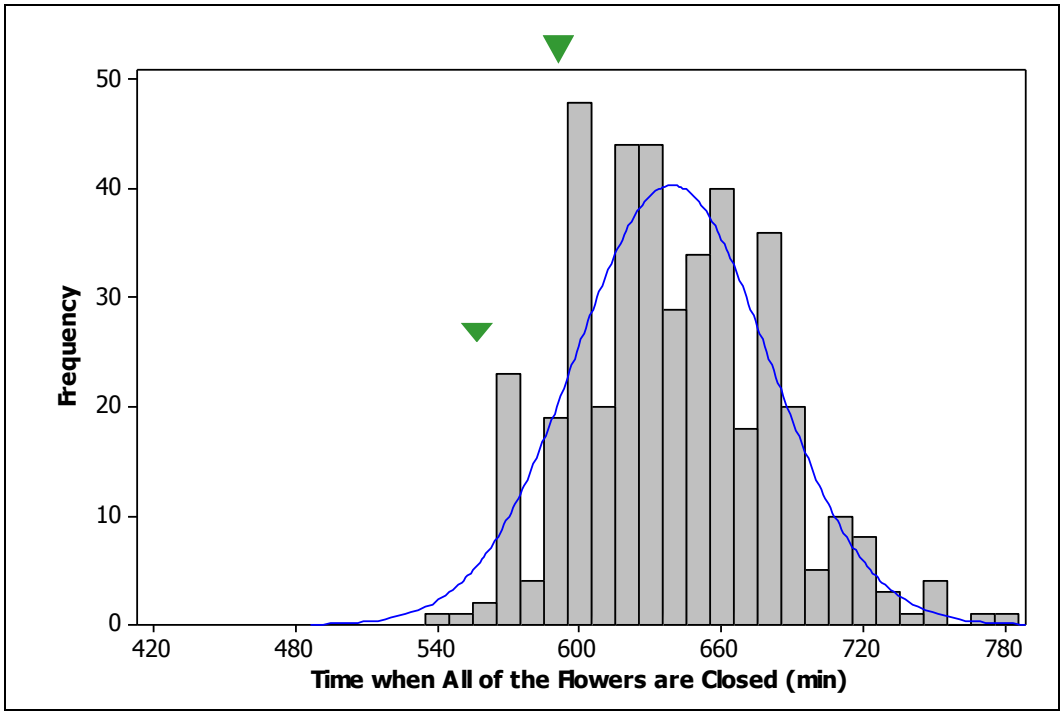

Figure 4. Frequency distribution of the time when all of the flowers are closed in 684 BC_1F_2 plants and their parents PSB-Rc82 and WAB56-125.

Polymorphism Between the Parental Varieties

A total of 217 SSR primer pairs were used to evaluate polymorphisms of the two parents (PSB-Rc82 and WAB56-125) used in this study. Among the 217 SSR markers surveyed, 164 (75.6%) showed polymorphism between the two parents used. From these 164 polymorphic SSR markers, only 85 SSR markers were utilized in selective genotyping of the 11 extreme heat-tolerant, 11 extreme heat-sensitive BC_1F_1 plants, and their parents. The selection of the 85 polymorphic SSR markers were based on the primers' distinct DNA banding patterns, and the genetic distance between markers that was set at between 10-20 cM along each chromosome. Information on each SSR primer, such as expected PCR product size, annealing temperature, and map position were obtained from Cornell University in the Gramene database (Gramene 2009).

High level of polymorphism in this study is attributed to the high genotypic variation between parents used which are *Indica (*PSB-Rc82) and *Oryza glaberrima*-derived (WAB56-125) rice genotypes. Generally, sufficient polymorphisms rely or exist with parents that are distantly related or genetically diverse (Anderson et al. 1993; Collard et al. 2003; Joshi and Nguyen 1993; Yu and Nguyen 1994).

QTL Analysis for the Early Morning Flowering (EMF) Traits

QTL analysis using selective genotyping revealed significant differences in the start of flower opening time among the 16 heat-sensitive and 27 heat-tolerant BC1F2 plants. The single marker analysis had shown the association of SSR markers RM233B ($p = 0.010$) on chromosome 5, RM321 ($p = 0.003$) on chromosome 9, and RM481 ($p = 0.019$) on chromosome 7 showed significant P value among the BC1F2 plants. These microsatellite markers can be possibly linked or associated to the start of flower opening time for the EMF trait in rice. Similarly, Thanh et al. (2010) using backcross population, identified QTL for starting time when the first spikelet opened (SOTb) on chromosome 5, with markers flanking RM249-RM440.

For the time of peak flowering, SSR markers RM556 ($p = 0.031$) on chromosome 8, RM574 ($p = 0.041$) and RM233B ($p = 0.002$) on chromosome 5, RM481 ($p = 0.026$) on chromosome 7, RM4 ($p = 0.034$) on chromosome 11, and RM321 ($p = 0.006$) on

chromosome 9 had significant P values among BC1F2 plants. These could be possibly linked to peak flowering time. Same markers were identified to be possibly linked to time when most of the flowers are closed and time when all of the flowers are closed. These results indicate that chromosomes 5, 7, 8, 9, and 11 were most likely the possible QTL locations for peak flowering time, time when most of the flowers are closed and time when all of the flowers are closed. Thanh et al. (2010) reported that related QTL was detected for peak flowering time on chromosome 5 in the region of RM249. In this study, the QTL region for SOTb and peak flowering time on chromosome 5 overlapped which could indicate the most likely location of flower opening time QTL in rice. This information could be used in determining the genetic basis of flower opening time in relation to early morning flowering.

Flower opening time varies among rice genotypes in response to high temperature. Kobayashi et al. (2010) showed that flower opening in the early morning can reduce sterility in rice, but it can be affected by weather such as air temperature (Nishiyama and Satake 1981; Imaki et al. 1983; Hoshikawa 1989; Jagadish et al. 2007, 2008; Nakagawa and Nagata, 2007). Similarly, Jagadish et al. (2007 and 2008) found that the flowers of rice varieties open earlier at high temperatures to avoid high midday temperatures.

For the time when most of the flowers are closed, SSR markers RM556 (p = 0.043) on chromosome 8, RM574 (p = 0.033) and RM233B (p = 0.001) on chromosome 5, RM481 (p = 0.036) on chromosome 7, RM4 (p = 0.030) on chromosome 11, and RM321 (p = 0.005) on chromosome 9, produced significant P values among BC1F2 plants through single marker analysis. These markers are possibly linked to QTL for time when most of the flowers are closed.

Single marker analysis (SMA) results revealed QTLs for time when all of the flowers are closed were possibly linked with SSR markers RM556 (p = 0.044) on chromosome 8, RM574 (p = 0.043) and RM233B (p = 0.001) on chromosome 5, RM481 (p = 0.027) on chromosome 7, RM4 (p = 0.034) on chromosome 11, and RM321 (p = 0.003) on chromosome 9.

In general, SSR markers RM556 on chromosome 8, RM574 and RM233B on chromosome 5, RM481 on chromosome 7, RM321 on chromosome 9, and RM4 on chromosome 11 were the most possibly linked to QTL for peak flowering time, time when most of the flowers are closed, and time when all of the flowers are closed. Therefore, the most likely sites of early morning flowering QTL are on chromosomes 5, 7, 8, 9 and 11.

CONCLUSION

The study aimed to identify quantitative trait loci (QTL) for early morning flowering (EMF) trait BC_1F_2 rice population and identify markers for marker-assisted selection. Genetic factors underlying EMF trait in rice were analyzed by selective genotyping of phenotypic extremes among 684 BC_1F_2 plants (derived from the 235 BC_1F_1 plants that was further derived from the cross PSB Rc82//PSB Rc82/WAB56-125) evaluated under glasshouse conditions were undertaken using single marker analysis (SMA). Four parameters were determined such as flower opening time or start time (FOT), peak of flowering (POF), flower mostly closed (FMC), and flower closed (FC) time using 684 BC_1F_2 plants.

Results revealed that SSR markers RM321 (p=0.003) on chromosome 9, RM233B (p=0.010) on chromosome 5, and RM481 (p=0.019) on chromosome 7 were possibly linked to QTL for FOT. While SSR markers RM556 on chromosome 8, RM574 and RM233B on chromosome 5, RM481 on chromosome 7, RM321 on chromosome 9, and RM4 on chromosome 11 were most possibly linked to QTL for POF, FMC, and FC. Results further indicate the most likely genomic regions of EMF QTL to be distributed on chromosomes 5, 7, 8, 9 and 11.

RECOMMENDATION

While this needs to be confirmed using a larger population size and other QTL mapping method/s, the results could already be useful for further fine mapping and eventually for marker-aided selection of EMF/heat escape rice varieties adapted to future warming climates.

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