QTL analysis for flowering time using backcross population between Oryza sativa Nipponbare and O. rufipogon

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In the near future, global average temperature is expected to increase due to the accumulation of greenhouse gases, and increased temperatures will cause severe sterility in many crop species. In rice, since wild species show high genetic variation, they may have the potential to improve the flowering characters of cultivars. In this study, we investigated flowering characters under natural conditions by comparing an Asian wild rice accession of Oryza rufipogon W630 (originated from Myanmar) with a Japanese rice cultivar, O. sativa Japonica cv. Nipponbare. Further, QTL analysis for days to heading (DH) and spikelet opening time (SOT: the time of day when the spikelet opens) was carried out using BC$_2$F$_8$ backcross population derived from the cross between them. Regarding DH, four QTLs were detected, and two of them were found to have wild alleles with strong effects leading to longer days to heading during the Japanese summer. These wild alleles may be used to produce late-heading cultivars that do not flower during the high summer temperatures anticipated in the future. For SOT, two parameters of SOTb (beginning time when the first spikelet opens) and SOTm (median time when 50% of the spikelets open) were recorded and the time differences from Nipponbare were investigated. Two QTLs on chromosomes 5 and 10 and two QTLs on chromosomes 4 and 5 were detected for SOTb and SOTm, respectively. The wild alleles were responsible for early spikelet opening time at all loci. If the wild alleles detected in this study have the same effects in the genetic background of other cultivars, they will be very useful in producing early-flowering rice cultivars that complete fertilization in the morning before the temperature rises.

Key words: heat avoidance, Oryza rufipogon, O. sativa, rice, spikelet opening time (SOT)

INTRODUCTION

Rice (Oryza sativa L.) is one of the most important crops and supports more than one-third of the world’s population (Khush, 1997). Rice is produced in the tropical countries and also during the summer seasons in temperate countries. The plants grow vegetatively under tropical condition but are very sensitive to heat during the heading and flowering stages. Satake and Yoshida (1978) reported that temperatures above 35°C led to severe spikelet sterility at flowering time. They further found that the flowering or opening spikelets were the most sensitive to high temperatures, and they observed normal fertilization in the spikelets that flowered 1 h before heat treatments. These results indicate that sterility is caused by poor anther dehiscence and low numbers of viable pollens and that heat insensitivity occurs after completion of fertilization (Satake and Yoshida, 1978; Matsui et al., 2000, 2001).

In 2007, the Intergovernmental Panel on Climate Change reported a 100-yr linear temperature trend (1906–2005) of 0.74°C (IPCC, 2007). They further estimated a global average temperature increase of 1.8–4.0°C for possible six scenarios at the end of the 21st century, suggesting that crop production will be reduced due to heat stress in the near future. According to Matsui et al. (2001) and Prasad et al. (2006), high temperatures signif-
iciently decreased spikelet fertility across all rice cultivars. Kobayashi et al. (2009) examined flower opening time among rice cultivars during the summer in Japan and found that most of the Japanese cultivars flowered after 1100 h, which was close to the time of the highest daytime temperature. These results strongly indicate that rice cultivars at flowering time will be highly susceptible to the projected future temperatures. Therefore, we should consider some biological improvements to prevent rice from flowering at high temperatures. One strategy is to change the heading period character. Since rice is a short-day plant, it may be possible to control the heading date by modifying photoperiod sensitivity. Another approach is to change the flowering time, i.e., the time of day when the spikelet opens. If rice plants flower early in the morning before the temperatures rise, they can complete their fertilization and produce seeds (Satake and Yoshida, 1978).

The time of day when flowering commenced was previously examined in rice cultivars and wild species (Sheehy et al., 2007). According to the study, flowering times varied more in wild rice species than in cultivars. This result indicates that wild species have the potential to improve the flowering characters of cultivars. Ishimaru et al. (2010) recently conducted a greenhouse experiment and reported that the introgression line with the early-morning flowering trait transferred from O. officinalis (CC genome) could avoid high-temperature-induced sterility. Among the AA genome wild rice species, O. rufipogon, the ancestral species of common cultivated rice (O. sativa) tends to flower earlier than cultivars (Sheehy et al., 2007), suggesting this wild species is also a candidate for modifying the flowering characters of cultivars. However, we do not know whether this character is controlled by single or multiple genes.

In this study, flowering characters under natural conditions were investigated by comparing a wild annual accession of O. rufipogon (W630) with a Japanese rice cultivar, O. sativa Japonica cv. Nipponbare. QTL analysis for heading date and flowering time was conducted using BC1F2 backcross population between O. rufipogon W630 and O. sativa Nipponbare. Regarding flowering time, we use a term of “spikelet opening time (SOT)” in this study, because we examined the time of day when the spikelet opens. This is the first report to estimate wild useful QTLs for SOT in rice.

MATERIALS AND METHODS

Plant materials A Japonica rice cultivar, O. sativa Nipponbare, and a wild annual accession of O. rufipogon from Myanmar (accession no. W630) were used in this study. The wild accession was provided by the National Institute of Genetics, Japan. The two species were crossed, and a total of 141 F2 plants were used to construct a molecular linkage map. Regarding flowering traits, evaluation was conducted with 161 BC1F2 plants between O. sativa Nipponbare (a recurrent parent) and O. rufipogon W630 (a donor parent). They were developed from 161 BC1F1 plants (derived from 18 independent BC1F1 plants) by the single-seed-descendant method. They theoretically contain 1/8 of the wild genome in the genetic background of O. sativa Nipponbare.

Construction of genetic linkage map using SSR markers DNA was extracted from 141 F2 plants using the potassium acetate method after Dellaporta et al. (1983). In total, 180 polymorphic SSR markers between O. sativa Nipponbare and O. rufipogon W630 were selected to examine the genotypes of F2 plants. Primer sequences of these SSR markers were obtained from the Gramene database (http://www.gramene.org/) by Liang et al. (2008). PCR was carried out in a 25-μl reaction mixture containing 0.2 μM of each primer, 100 μM of each dNTP, 10 mM Tris-Cl (pH 8.3), 1.5 mM MgCl2, 0.1% Triton X-100, 25–50 ng of template DNA, and 1 unit of Taq DNA polymerase (TOYOBO Co., Ltd., Japan). Amplification was performed with PTC100 programmable thermal controller (MJ Research Inc., USA) at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50–60°C (depending on the annealing temperature for each microsatellite marker) for 1 min, 72°C for 2 min, and a final extension of 72°C for 5 min. Amplified products were electrophoresed in 4% polyacrylamide denaturing gel with 0.5 × TBE buffer. The microsatellite banding patterns were visualized using the silver staining method as described by Panaud et al. (1996). After scoring the marker genotypes of 141 F2 plants, linkage groups and marker orders were determined using MapMaker ver. 2.0 software (Lander et al., 1987). The Kosambi mapping function was used to transform the recombination frequency to genetic distances (cM) (Kosambi, 1944).

Flowering trait evaluation Flowering traits were evaluated for 161 BC1F2 plants and their parents at Kobe University, Kobe, Japan (34°43′28″N, 135°14′8″E) in 2008. Their seeds were soaked on May 2, and the germinated seeds were sown in the glasshouse on May 7, 2008. The pots containing plants with flag leaves were moved outside the glasshouse, and allowed to flower under natural conditions. Days to heading (DH) were recorded as the number of days from seed soaking to first panicle heading.

To investigate spikelet opening time (SOT), at least three panicles (with more than 10 flowering spikelets) per plant were examined from August 11 to September 4 (except on some rainy days). For each panicle, all opening spikelets were counted with a marking pen every 15 min from 7:45 AM to 1:30 PM. The beginning time when the first spikelet opened (SOTb) and the median time

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when 50% of the spikelets opened (SOTm) were recorded. These values were calculated further as the average values of the panicles examined. Additional parental plants of *O. sativa* Nipponbare and *O. rufipogon* W630 were continuously prepared to keep flowering plants during the entire period of trait evaluation. Since the backcross population was produced in the genetic background of *O. sativa* Nipponbare, their SOT was much closer to that of Nipponbare than that of wild. In general, the opening times of Nipponbare varied according to the date and weather. Therefore, the relative earliness of spikelet opening time for each plant was evaluated as the time difference from *O. sativa* Nipponbare as follows: (relative earliness) = (time of Nipponbare) – (time of the backcross plant). In this study, two parameters were calculated to determine the relative earliness of SOTb and SOTm. These values were used in the QTL analysis for SOT character from wild rice.

To examine the climatic conditions affecting spikelet opening, outdoor temperature and humidity were recorded every 5 min during the investigation period by using a model RS-12 thermo recorder (Espec Mic Co., Japan).

QTL analysis for flowering traits DNA samples were prepared from 161 BC2F8 plants according to the method of Dellaporta et al. (1983). Marker genotypes at 180 SSR loci were determined using the same methods as mentioned above. The marker data were combined with the trait data, and QTL analysis for DH and SOT was carried out. Putative QTLs were estimated by composite interval mapping (CIM) using WinQTL Cartographer ver. 2.5 software (Wang et al., 2007). The optimal log of odds (LOD) threshold values obtained from WinQTL Cartographer (with the permutation value set at 1000) were used to determine the presence of a putative QTL. The percentages of variation explained by the QTL and the additive effect were also estimated using the software.

**RESULTS AND DISCUSSION**

Construction of molecular linkage map between *O. sativa* Nipponbare and *O. rufipogon* W630 Marker genotypes at 180 polymorphic SSR loci were examined using 141 F2 plants between *O. sativa* Nipponbare and *O. rufipogon* W630. Based on these data, linkage analysis was carried out using MapMaker ver. 2.0 (Lander et al., 1987), and an interspecific linkage map was constructed between *O. sativa* Nipponbare and *O. rufipogon* W630. The marker orders on the chromosomes were the same as those reported in the Gramene database (http://www.gramene.org/). Total map size was 1362 cM with an average marker interval of 8.2 cM; the largest marker interval was 24.6 cM on chromosome 2. The highest density of markers occurred on chromosome 2 (22 markers) and the lowest occurred on chromosome 5 and 10 (11 markers). This finding suggests that many microsatellite markers, which were designed based on the nucleotide sequences of rice cultivars, can be used with *O. rufipogon* and applied for map-based studies, such as gene tagging and interspecific QTL analysis.
QTL analysis for days to heading  The number of days from seed soaking (May 2, 2008) to heading were examined in 161 BC2F8 plants and their parents under natural summer conditions at Kobe University, Japan. Except for one plant with no heading, 160 plants were scored for DH; the frequency distribution in the backcross population is shown in Fig. 1. Many plants with transgressive values were observed in the backcross population.

QTL analysis for DH was carried out with marker genotype data at 180 microsatellite loci using the backcross population. As a result, four QTLs were detected (Table 1 and Fig. 2). Among the detected QTLs, one on chromosome 3 (nearest marker RM571) and one on chromosome 8 (nearest marker RM25) explained 43.7% and 19.0%, respectively, of the total phenotypic variance in the population. The wild alleles at these loci were found to cause longer days to heading during the Japanese summer. In the same regions, two QTLs for heading date (Hd6 and Hd5) were reported previously by Yamamoto et al. (2000) and Lin et al. (2003) using backcross progenies between O. sativa Japonica Nipponbare and Indica Kasalath. At both loci, Kasalath alleles caused an increase (> 20 days) in days to heading under 13.5- to 14-h day length conditions. These results strongly indicate that the two QTLs detected in this study

Table 1. Putative QTLs for days to heading (DH) and spikelet opening time (SOTb: beginning time, SOTm: median time) detected in BC2F8 backcross population between O. sativa Nipponbare and O. rufipogon W630

<table>
<thead>
<tr>
<th>Trait</th>
<th>Chr.</th>
<th>QTL location</th>
<th>Nearest marker</th>
<th>Source*</th>
<th>LOD score</th>
<th>PVb (%)</th>
<th>Additive effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>2</td>
<td>RM221-RM6</td>
<td>RM6</td>
<td>W630</td>
<td>4.11</td>
<td>4.99</td>
<td>2.77 d</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>RM135-RM571</td>
<td>RM571</td>
<td>W630</td>
<td>26.47</td>
<td>43.72</td>
<td>8.94 d</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>RM319-RM25</td>
<td>RM25</td>
<td>W630</td>
<td>11.41</td>
<td>18.96</td>
<td>5.50 d</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>RM484-RM228</td>
<td>RM228</td>
<td>W630</td>
<td>6.84</td>
<td>8.80</td>
<td>3.90 d</td>
</tr>
<tr>
<td>SOTb</td>
<td>5</td>
<td>RM249-RM440</td>
<td>RM440</td>
<td>W630</td>
<td>5.43</td>
<td>27.21</td>
<td>0.42 h</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>RM171-RM496</td>
<td>RM484</td>
<td>W630</td>
<td>4.83</td>
<td>28.55</td>
<td>0.48 h</td>
</tr>
<tr>
<td>SOTm</td>
<td>4</td>
<td>RM303-RM255</td>
<td>RM255</td>
<td>W630</td>
<td>3.04</td>
<td>14.66</td>
<td>0.29 h</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>RM249</td>
<td>RM249</td>
<td>W630</td>
<td>3.39</td>
<td>20.50</td>
<td>0.37 h</td>
</tr>
</tbody>
</table>


Fig. 2. Chromosome positions of putative QTLs controlling days to heading (DH) and spikelet opening time (SOTb: beginning time, SOTm: median time) detected in BC2F8 backcross population between O. sativa Nipponbare and O. rufipogon W630. QTL positions are represented as bars with the LOD peak shown by arrows. Black and white bars indicate the allele effects of W630 and Nipponbare to increase the trait values, respectively.
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would be the same loci of *Hd6* and *Hd5* that control days to heading under long-day conditions. The wild alleles at these loci may change the heading period in temperate zones under long-day conditions. If future mid-summer temperatures are high enough to cause pollen sterility, the wild alleles could be used to produce late-heading cultivars that flower in autumn. Since these cultivars would avoid flowering at high temperatures during the summer, fertilization would be normal.

Fig. 3. Relationship between climatic conditions and spikelet opening time (SOTb: beginning time, SOTm: median time) observed for *O. sativa* Nipponbare and *O. rufipogon* W630. (a), (b): Temperature and SOTb. (c), (d): Temperature and SOTm. (e), (f): Humidity and SOTb. (g), (h): Humidity and SOTm. ns: Not significant. **: Significant at 1% level.
Spikelet opening times for cultivated and wild rice under natural conditions in Japan  To investigate the flowering time of day for cultivated and wild rice under natural summer conditions, spikelet opening times (SOTb and SOTm) of *O. sativa* Nipponbare and *O. rufipogon* W630 were examined from August 11 to September 4. The flowering plants were prepared continuously during the evaluation period. For both of them, more than three panicles were observed daily; however, we could not obtain flowering data on five rainy days (August 21, 23, 28 and 30, and September 3). Under all climatic conditions, *O. rufipogon* W630 flowered earlier than *O. sativa* Nipponbare. Since outdoor temperature and humidity were recorded during the entire evaluation period, these climatic conditions at two kinds of spikelet opening times (SOTb and SOTm) were extracted (Table 2). Fig. 3 shows the relationship between climatic conditions (temperature and humidity) and spikelet opening times (SOTb and SOTm) for *O. rufipogon* W630 and *O. sativa* Nipponbare. The analysis of flowering temperature showed significant negative correlations in SOTb (*r* = −0.535) and SOTm (*r* = −0.691) for W630, whereas no correlations were observed for Nipponbare. On the other hand, a significant positive correlation was only observed between humidity and SOTb (*r* = 0.564) for Nipponbare. These results indicate that in W630, spikelet opening is dependent on the temperature, but in Nipponbare, spikelet opening seems to be controlled by many other factors under natural climatic conditions.

### Evaluation of SOT values for backcross plants

During the evaluation period (August 11–September 4), various climatic conditions, such as rainy, cloudy, sunny, humid, and hot were observed, but wild rice always flowered earlier than Nipponbare. On sunny days, rapid temperature increase was observed after sunrise, and the peak flowering times of W630 were 2–3 h earlier than those of Nipponbare. However, under some conditions, their peak flowering times became relatively close (about 1 h). For example, Nipponbare flowered earlier than usual on days after the rain, and W630 delayed flowering on days with cool temperatures in the morning. These suggest that wild early flowering characters are well-reflected on the days giving large flowering time differences between W630 and Nipponbare. Therefore, in this study, we assume that reliable flowering data controlled by temperature are obtained on the day when the peak flowering time (SOTm) of W630 is more than 2 h earlier than that of Nipponbare.

During the same evaluation period, SOTb and SOTm were also examined for BC$_2$F$_6$ plants. The relative earliness of spikelet opening time was measured as the time difference from *O. sativa* Nipponbare. Flowering data collected on the desirable days mentioned above were selected, and SOT values were obtained for 77 BC$_2$F$_6$ plants. Fig. 1 shows the distribution of the relative earliness of SOTb and SOTm in the backcross population. The relative values of SOTb and SOTm ranged from 0.41 to 3.42 h and from −1.15 to 1.80 h, respectively. These continuous distributions showed that SOT characters were controlled by many genes.

### QTL analysis for SOT

Using the relative earliness values of the backcross plants, QTL analysis for SOT was carried out by composite interval mapping (Table 1 and Fig. 2). For SOTb, two QTLs were estimated on chromosomes 5 and 10 with LOD scores of 5.43 and 4.83, respectively. They each explained about 28% of the phenotypic variance, and the wild alleles were responsible for the early opening time at both loci. Two QTLs were also detected for SOTm on chromosomes 4 and 5 in the regions of RM303–RM255 and RM249, respectively. In addition, the additive effects of single wild alleles at these loci were 0.29 and 0.37 h, respectively. These results indicate that the homozygous wild alleles at both loci have potential to promote flowering more than 0.5 h earlier. Interestingly, the QTL regions for SOTb and SOTm on chromosome 5 overlapped. Probably they may share the same locus having association with temperature. Since the whole-panicle flowering tendency is better explained by SOTm than by SOTb, the wild alleles responsible for early SOTm will be useful in controlling cultivar flowering characters depending on the temperature.

Spikelet sterility varied in response to high temperatures in rice (Prasad et al., 2006; Jagadish et al., 2007). If the wild alleles detected in this study have the same effects in the genetic background of other cultivars, they will be very useful for changing flowering times to avoid high temperatures in the future.

This is the first report on QTL analysis for SOT, and the genetic analysis of this character has just started. Therefore, it is necessary to produce near-isogenic lines for these QTLs to confirm the wild allele effects. In addition, we need to assess spikelet fertility and sterility under high-temperature conditions.

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Table 2. Average, minimum and maximum values of temperature and humidity at spikelet opening times, SOTb and SOTm, observed for *O. sativa* Nipponbare and *O. rufipogon* W630^a^.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Trait</th>
<th>Temperature (°C)</th>
<th>Humidity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>Min.</td>
</tr>
<tr>
<td>Nipponbare</td>
<td>SOTb</td>
<td>31.2</td>
<td>26.2</td>
</tr>
<tr>
<td></td>
<td>SOTm</td>
<td>32.0</td>
<td>26.1</td>
</tr>
<tr>
<td>W630</td>
<td>SOTb</td>
<td>29.1</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>SOTm</td>
<td>30.0</td>
<td>26.3</td>
</tr>
</tbody>
</table>

^a^: Data were taken on 20 days (from August 11 to September 4 except five rainy days).
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