**RESEARCH PAPER**

**qEMF3**, a novel QTL for the early-morning flowering trait from wild rice, *Oryza officinalis*, to mitigate heat stress damage at flowering in rice, *O. sativa*

Hideyuki Hirabayashi¹, Kazuhiro Sasaki²,³,⁴, Takashi Kambe¹, Ritchel B. Gannaban³, Monaliza A. Miras⁵, Merlyn S. Mendioro⁵, Eliza V. Simon³, Patrick D. Lumanglas³, Daisuke Fujita³,⁴, Yoko Takemoto-Kuno¹, Yoshinobu Takeuchi¹, Ryota Kaji³, Motohiko Kondo¹, Nobuya Kobayashi¹,²,³, Tsugufumi Ogawa⁷, Ikuo Ando¹, Krishna S. V. Jagadish³ and Tsutomu Ishimaru¹,²,³,‡‡

¹ NARO Institute of Crop Science, NARO, 2-1-18 Kannondai, Tsukuba, Ibaraki 305-8518, Japan
² Japan International Research Centre for Agricultural Sciences (JIRCAS), 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan
³ International Rice Research Institute (IRRI), DAPO Box 7777, Metro Manila, The Philippines
⁴ The University of Tokyo, Graduate School of Agricultural and Life Sciences, Institute of Sustainable Agro-ecosystem Services (ISAS), 1-1-1 Midori, Nishitokyo, Tokyo 188-0002, Japan
⁵ University of the Philippines, Los Baños, Laguna, The Philippines
⁶ NARO Tohoku Agricultural Research Centre (TARC), NARO, Shimo Furumichi, Daisen, Akita 014-0102, Japan
⁷ Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen kihanadai nishi, Miyazaki, Miyazaki 889-2192, Japan

* Present address: Niigata Crop Research Centre, Nagakura-machi 857, Nagaoka, Niigata, 940-0826, Japan
† Present address: Kyushu University, faculty of agriculture, Hakozaki 6-10-1, Higashi-ku, Fukuoka, 812-8581, Japan
‡ These authors contributed equally to this work.
‡‡ To whom correspondence should be addressed. E-mail: t.ishimaru@irri.org

Received 24 September 2014; Revised 23 October 2014; Accepted 24 October 2014

**Abstract**

A decline in rice (*Oryza sativa* L.) production caused by heat stress is one of the biggest concerns resulting from future climate change. Rice spikelets are most susceptible to heat stress at flowering. The early-morning flowering (EMF) trait mitigates heat-induced spikelet sterility at the flowering stage by escaping heat stress during the daytime. We attempted to develop near-isogenic lines (NILs) for EMF in the *indica*-type genetic background by exploiting the EMF locus from wild rice, *O. officinalis* (CC genome). A stable quantitative trait locus (QTL) for flower opening time (FOT) was detected on chromosome 3. A QTL was designated as qEMF3 and it shifted FOT by 1.5–2.0 h earlier for cv. Nanjing 11 in temperate Japan and cv. IR64 in the Philippine tropics. NILs for EMF mitigated heat-induced spikelet sterility under elevated temperature conditions completing flower opening before reaching 35°C, a general threshold value leading to spikelet sterility. Quantification of FOT of cultivars popular in the tropics and subtropics did not reveal the EMF trait in any of the cultivars tested, suggesting that qEMF3 has the potential to advance FOT of currently popular cultivars to escape heat stress at flowering under future hotter climates. This is the first report to examine rice with the EMF trait through marker-assisted breeding using wild rice as a genetic resource.

**Key words:** Early-morning flowering (EMF), flower opening time (FOT), global warming, heat stress, quantitative trait locus (QTL), rice (*Oryza sativa* L.), spikelet sterility.

Abbreviations: BFOT, beginning of flower opening time; EMF, early-morning flowering; FOT, flower opening time; PFOT, peak of flower opening time.

© The Author 2014. Published by Oxford University Press on behalf of the Society for Experimental Biology.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Introduction

Rice (Oryza sativa L.) is one of the most important staple food crops for nearly half the world’s population (Carriger and Vallee, 2007). Predicted future climate change is expected to have a negative impact on global rice production. Maximum and minimum daily temperatures, and the number of hot days and warm nights in a year, are estimated to increase over most land areas (IPCC, 2013). In addition, climate variability is predicted to increase, leading to frequent episodes of heat stress, often coinciding with key developmental stages in crops, such as flowering. Seasonal temperatures in the tropics and subtropics by the end of the 21st century are predicted to exceed the most extreme seasonal temperatures recorded in the past (Battisti and Naylor, 2009). Breeding crops able to cope with future climate change is a pressing requirement to feed the growing population in the era of global warming.

High temperatures (>35°C) at flowering cause spikelet sterility in rice (Satake and Yoshida, 1978; Matsui et al., 1997). Heat stress for 1 h induces spikelet sterility if it coincides with flowering (Jagadish et al., 2007). Exposure of flowering spikelets to heat stress results in the failure of anther dehiscence and the reduced number of germinating pollen grains on the stigma (Satake and Yoshida 1978; Matsui et al., 2001a; Matsui and Omasa, 2002; Jagadish et al., 2010). Spikelet sterility positively correlates with a yield reduction in experimental paddy fields (Kim et al., 1996; Oh-e et al., 2007), in line with the yield reduction caused by heat-induced spikelet sterility observed in tropical Asia (Osada et al., 1973) and Africa (Matsushima et al., 1982). In Bangladesh, eastern India, southern Myanmar, and northern Thailand, the flowering and early grain-filling stages of rice are predicted to coincide with high-temperature conditions, according to the local cropping patterns and crop calendars (Maclean et al., 2002; Wassmann et al., 2009). In the Jianghan Basin in China, significant losses in seed set were observed in local hybrid rice because of hot and humid conditions during flowering (Tian et al., 2010). Damage to rice productivity because of heat stress at flowering has also been reported in temperate regions of Japan: in comparison with normal summers, spikelet sterility was significantly higher by 15–20% in the summer of 2007, when maximum temperatures reached a record level of 40°C during the flowering stage (Hasegawa et al., 2011). Thus, heat stress at flowering poses a real threat to sustained rice production not only in tropical and subtropical regions, but also in temperate regions. Existing breeding programmes are challenged to develop rice that would be heat-resilient at flowering and could be grown across temperate, tropical, and subtropical regions.

To mitigate heat-induced spikelet sterility, two strategies have been proposed. One is to develop cultivars that shed larger numbers of pollen grains or produce pollen grains able to germinate at high temperatures. At the flowering stage, rice cultivars have different tolerance to high temperatures (Satake and Yoshida, 1978; Matsui et al., 2001b; Prasad et al., 2006; Jagadish et al., 2008). A highly heat-tolerant cultivar, N22 (Satake and Yoshida, 1978; Mackill et al., 1982; Jagadish et al., 2010), has the tolerance allele qHTSF4.1, a QTL on chromosome 4, as a major genetic factor (Ye et al., 2012). Another strategy, which is less explored, is to breed cultivars that escape heat at flowering because of their early-morning flowering (EMF) trait (Satake and Yoshida, 1978). Spikelets are highly susceptible to heat stress at flowering; however, they remain fertile when flowering occurs 1 h prior to heat stress, because fertilization is completed within 1 h after the onset of flowering (Satake and Yoshida, 1978). The EMF strategy has been used to produce an introgression line, EMF20, with the EMF trait transferred from wild rice, O. officinalis, into the genetic background of O. sativa cv. Koshihikari (Ishimaru et al., 2010). EMF20 flowered a few hours earlier than Koshihikari, which reduced heat-induced sterility at flowering at elevated temperatures in a greenhouse test (Ishimaru et al., 2010).

Within the genus Oryza, the flower opening time (FOT) ranges widely from early morning to midnight. There are many accessions in wild rice with the EMF trait (Sheehy et al., 2005). CG14 (O. glaberrima) has been reported as an EMF cultivar (Jagadish et al., 2008). By using backcrossed inbred lines derived from a cross between O. rufipogon and O. sativa, QTLs for FOT have been detected on chromosomes 4, 5, and 10 (Thanh et al., 2010). The O. rufipogon alleles of all QTLs theoretically contributed to the 30 min advancement of FOT in O. sativa (Thanh et al., 2010). However, the effect of the QTLs detected is yet to be proven to be materialized as a near-isogenic line (NIL) for EMF. Currently, there is no material available from ongoing rice breeding programmes that could be used to introduce a heat-escape trait to mitigate heat-induced spikelet sterility at flowering. In addition, there has been no systematic analysis of flowering dynamics of a large number of popular local cultivars occupying large areas in rice-growing regions, which would help to evaluate their heat-escape potential.

Hence, our work had three major objectives: (i) to identify QTLs for EMF and to develop NILs for EMF by using molecular markers and exploiting the EMF trait of the wild rice O. officinalis; (ii) to characterize the flowering pattern conferred by QTLs identified for EMF under various high-temperature regimes and at different geographical locations; and (iii) to quantify the FOT of popular cultivars grown across tropical and subtropical regions by systematic observation of their daily flowering patterns.

Materials and methods

Plant materials for QTL analysis

Rice plants were grown at the NARO Institute of Crop Science (NICS), Tsukuba (36° 02’N, 140° 10’E), Ibaraki, Japan. The introgression line, EMF20, was crossed with Nanjing 11 (Supplementary Figure S1A) because of the similar heading date in that location. The F2 population and self-pollinated F2 lines were derived from a cross between EMF20 and Nanjing 11 (Supplementary Figure S1A). The F2 seeds (in 2007) and F3 seeds (in 2008) were sown in seeding trays and 20-day-old seedlings were transplanted (146 F2 plants and 10 F3 plants into 250-cm2 pots, one plant per pot). As a basal dressing, a controlled-release fertilizer was applied in both
experiments, which contained N (0.5 g), P₂O₅ (2.3 g), and K₂O (2.2 g) in each pot. Plants were kept free from pests and diseases by preventive application of chemicals.

FOT of F₂ and F₃ plants
To reduce the phenological variation, a short-day treatment (light conditions for 9 h) was imposed on Nanjing 11, EMF20, F₂ plants, and F₁ lines for 2 weeks before the panicle-initiation stage. The heading date among all the above entries was narrowed down to just 9 days (from 1 to 9 August 2007). In addition, solar radiation and air temperature are known to strongly affect the daily flowering pattern (Kobayashi et al., 2010; Julia and Dingkuhn, 2012), and hence to reduce the variation induced by environmental factors, FOT was recorded only on sunny days. Opened spikelets were visually counted every 20 min using two panicles per plant per day and in three plants each from EMF20, Nanjing 11, and 146 F₂ populations. The beginning of FOT (BFOT, the time when the first spikelet opened on a given day) and the peak of FOT (PFOT, the time when the largest percentage of spikelets were open on a given day) were recorded for at least 2 days in 2007 for each individual F₂ plant; BFOT of 10 F₃ progenies of each F₂ plant was recorded daily in 2008 for 4 days. EMF20 and Nanjing 11 were stagger-sown every 3 days to make their genotypes cover the entire period of heading dates of the F₂ and F₃ plants. The BFOT and PFOT data were used for QTL analysis.

DNA extraction, map construction, and QTL analyses
Plant DNA was extracted from young leaves of the F₂ plants and their parents using the CTAB method (Murray and Thompson, 1980). A genetic map was developed using 154 simple sequence repeat (SSR) markers (McCouch et al., 2002). DNA amplification was performed for 35 cycles of 94°C (1 min), 55°C (2 min), and 72°C (3 min), and a final extension at 72°C for 7 min (PTC100, BioRad) using Taq DNA Polymerase (Thermo Fisher Scientific, Inc.). Amplified DNA products were electrophoresed in a 3.0% agarose gel in 0.5×TBE buffer. Analysis of linkage between SSR markers and linkage map construction for QTL analysis were performed with MAPMAKER/EXP 3.0 software (Lander et al., 1987). QTL analysis was performed with composite interval mapping analysis in the WinQTL Cartographer 2.5 software (Wang et al., 2007). The optimal log of odds (LOD) threshold values obtained with the permutation value set at 1000 by WinQTL Cartographer were used to determine the presence of a putative QTL, the percentages of variation explained by the QTL, and the additive effect.

Segregation analysis and development of NILs for the EMF locus
To confirm the effect of the QTL detected (qEMF3), segregation analysis for BFOT and PFOT was performed by using the BC₂F₂ population. By using SSR markers in the qEMF3 region (Fig. 2A), BC₁F₂ plants were classified into EMF20-homozygous, Nanjing 11-homozygous, and heterozygous. BFOT and PFOT were recorded from 10 plants of each classified group mentioned above and Nanjing 11 (as described in the section ‘FOT of F₂ and F₃ plants’) from late July to mid-August 2009 in Japan. To develop NILs for qEMF3, EMF20-homozygous plants for qEMF3 were selected from BC₂F₂ plants by using the same SSR markers used in the segregation analysis. From these BC₂F₂ plants, a plant that was Nanjing 11-homozygous for other chromosome segments was selected and used as a NIL (Nanjing 11+qEMF3) for the following experiments. The heading date of BC₂F₂ plants with heterozygous qEMF8 was 2 months later than that of the recurrent parent, Nanjing 11, resulting in a failure to proceed to the BC₂F₃ generation.

Nanjing 11 is well adapted to temperate rice-growing regions. To facilitate field testing of the EMF trait in heat-vulnerable regions in the tropics and subtropics, qEMF3 was transferred to IR64, a popular indica-type cultivar adapted for tropical and subtropical regions (Table 3; Khush, 1987). To develop IR64+qEMF3, Nanjing 11+qEMF3 and IR64 were used as the donor and recurrent parent, respectively (Supplementary Figure S1B). A foreground screening of the BC₁F₂, BC₂F₁, and BC₂F₂ populations was conducted with the flanking SSR markers RM 14360, RM 14374, and RM 14394 to confirm the qEMF3 genotype (Fig. 2A). A total of 32 SSR markers showing polymorphism between IR64 and Nanjing 11 were used for a background survey of 98 BC₁F₂ plants. An individual plant with a clear IR64 background (except in the qEMF3 region) was selected and self-pollinated to proceed to the BC₂F₁ generation. BC₂F₂ plants with the EMF20 alleles at flanking markers for qEMF3 were selected and advanced to BC₂F₁ to obtain seeds of NILs for qEMF3 in the IR64 genetic background (IR64+qEMF3). The flowering patterns of IR64 and IR64+qEMF3 were evaluated under flooded-field conditions in the wet season of 2013 (for 4 days in the middle of September) and in the dry season of 2014 (for 3 days in the middle of February) at the International Rice Research Institute (IRRI), Los Baños, the Philippines (14° 11’N, 121° 15’E). Plants were grown as described by Fujita et al. (2013). Spikelets that opened during the experiment were marked using fine-tipped pens every 30 min, from 07:00 until 13:30. Four panicles from four individual plants were collected from each genotype one day. The time from dawn to 50% of the FOT (T50) was calculated as described below to compare FOTs between IR64 and IR64+qEMF3.

Greenhouse experiment with elevated day temperatures
Nanjing 11 and Nanjing 11+qEMF3 were used to observe flowering patterns and to test sterility by using the method of Ishimaru et al. (2010). The experiment was conducted in 2010 in a glasshouse with clear-paned windows at NICS. Air temperature was manually increased by 2.0–3.5°C per hour after 08.00h. Maximum temperature reached ~40°C around noon. Air temperature was monitored every 15 min with dataloggers (SK-L200TH II, Sato Keiryoki Mfg., Tokyo, Japan). Opened spikelets were marked with fine-tipped pens every hour. All pots were returned to the open-windowed greenhouse after marking. Sterile spikelets were manually counted at maturity.

Heat tolerance test in an environmentally controlled growth chamber
Nanjing 11, Nanjing 11+qEMF3, IR64, and IR64+qEMF3 were grown in 10-l pots. Plants were moved into walk-in growth chambers at NICS. Heat tolerance test were marked, and after the end of the treatment period, all pots were returned to the non-stress greenhouse conditions. No spikelet flowering was observed before and after the temperature treatment. Sterility of the marked spikelets was estimated by manually counting the filled and unfilled spikelets.

Response of IR64 and IR64+qEMF3 to elevated high temperatures
Twelve seeds were regularly and circumferentially sown in 10-l pots using the method of Satake (1972). Plants were trimmed to the main stem once a week and grown in a greenhouse until the flowering stage. The flowering pattern was investigated in the walk-in chamber under three settings of temperature regime: (1) 25°C at 06.00, linearly increasing to 40°C by 14.00, then gradually decreasing to 25°C by 19.00; (2) 30 °C at 06.00, linearly increasing to 40°C by 
by 14.00 h, then gradually decreasing to 30°C by 19.00 h; and (3), as (1) except that the temperature maximum (40°C) was reached at 12.00 h. The lights were on from 06.00 h until 19.00 h; relative humidity was constant (60%) during the light period. Two or three panicles with the same heading date were selected per pot. Opened spikelets were marked manually every 30 min after 06.00 h. Eight panicles from three or four pots were used per genotype per day. Actual values of temperature and humidity were monitored every 15 min with data loggers. After each temperature treatment, pots were returned to the greenhouse. To acclimate the plants to chamber conditions before the experiments, new pots were moved from the walk-in chamber maintained at 25°C or 30°C to the walk-in chamber used for this experiment at 19.00 h. No flowering was observed before 06.00 h. The experiment was repeated for 3 days for each temperature regime.

**Plant material and determination of FOT of popular local cultivars**

The flowering patterns of cultivars listed in Table 3 were investigated in a naturally illuminated greenhouse at IRRI. We selected 23 popular cultivars from Southeast Asia (five cultivars), South Asia (five cultivars), Africa (eight cultivars), and Latin America (five cultivars) on the basis of nomination by the breeders in the CGIAR centres (IRRI, AfricaRice, and CIAT; International Centre for Tropical Agriculture) and at Tamil Nadu Agricultural University (Southern India). Nanjing 11, Nanjing 11+ qEMF3, and CG14 (an EMF cultivar of *O. glaberrima*; Jagadish et al., 2008) were also used. Nanjing 11+ qEMF3 was used as a control for the EMF trait. Plants were grown during the dry seasons of 2012 and 2013 at IRRI. Two 3-week-old seedlings were transplanted into a 10-l pot filled with 7.0 kg of clay loam soil with a basal dressing of N (0.42 g), P₂O₅ (0.42 g), and K₂O (0.42 g) in each pot. Additional N (0.63 g) was applied as top-dressing at 2 and 4 weeks after transplanting. Plants were grown under flooded conditions throughout the experiments. Standard plant protection measures at IRRI were followed; plants did not have any pest or disease damage. Experiments were conducted only on sunny days. More than three panicles (one panicle per plant) were used per genotype per day. Opened spikelets were marked every 30 min from 07.30 (before the start of flowering) to 14.00 (until the end of flowering) using fine-tipped pens. Heading dates for different cultivars ranged from 22 February to 3 May. Nanjing 11+ qEMF3 was sown at weekly intervals from December until March to synchronize its heading date with that of each cultivar. Air temperature and relative humidity were monitored every 30 min from 07.30 to 14.00 with data loggers (Supplementary Table S1). The experiment was repeated for at least three consecutive days for each genotype.

**Calculation of the beginning, peak, and end of FOT**

FOT was included in the ‘time (hour) after dawn’ criterion because of the wide range of genotype heading dates and difference in natural day-length between wet and dry seasons. The exact time of dawn was calculated using the Koyomi Station software on the website of the Ephemeris Computation Office, Public Relations Centre, National Astronomical Observatory of Japan (http://eco.mtk.nao.ac.jp/koyomi/index.html.en). The time of dawn changed from 06.49 to 06.05 during the experimental period (from 22 February to 3 May). The flowering pattern was presented as the percentage of opened spikelets on each day (y-axis) plotted against time after dawn (x-axis) (Supplementary Figure S2A). Curves were fitted by probit analysis using the R program (ver. 2.15.1) based on the cumulative percentage of opened spikelets (Supplementary Figure S2B and S2C). The times from dawn to 10% of the FOT, to 50% of the FOT, and to 90% of the FOT were calculated and defined as T10, T50, and T90, respectively. T10, T50, and T90 were also calculated using the R program.

**Statistical analyses**

The differences of means were analysed using the t-test or Tukey–Kramer test implemented in the R package (ver. 2.15.1).

**Results**

**Identification of QTLs for EMF**

In 2007, the mean BFOT was 07.00 in EMF20 and 08.10 in Nanjing 11. The BFOT and PFOT of the 146 F₂ plants from a cross between EMF20 and Nanjing 11 showed a normal frequency distribution (Fig. 1A, B). By using composite interval mapping analysis with 154 SSR markers and the 2007 data, we identified a significant QTL for BFOT (LOD score of 3.3) on chromosome 8, which explained 12.3% of the phenotypic variation (Table 1). For PFOT, two QTLs were identified on chromosomes 3 (LOD score of 3.8) and 8 (LOD score of 5.2), which explained 12.9 and 13.0% of the phenotypic variation, respectively (Table 1). In 2008, BFOT of the 146 F₃ lines derived from F₂ individuals was distributed...
normally (Fig. 1C). QTLs for BFOT were identified on chromosome 3 (LOD score of 8.4; 19.7% of phenotypic variation explained), chromosome 6 (LOD score of 2.5; 4.8% of phenotypic variation), and chromosome 8 (LOD score of 5.6; 12.9% of phenotypic variation) (Table 1). In both the F$_2$ and F$_3$ populations, the EMF20 alleles of QTLs on chromosomes 3 and 8 advanced FOT to early in the morning; these two QTLs were designated as qEMF3 and qEMF8 (because of the QTL for Early-Morning Flowering), respectively.

### Table 1. QTLs for the beginning and peak of FOT

<table>
<thead>
<tr>
<th>Year</th>
<th>Trait</th>
<th>Chromosome</th>
<th>Nearest- Marker</th>
<th>LOD score</th>
<th>AE$^a$</th>
<th>VE$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>BFOT</td>
<td>3</td>
<td>RM14407</td>
<td>2.4</td>
<td>−16.0</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>RM22459</td>
<td>3.3</td>
<td>−17.6</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>PFOT</td>
<td>3</td>
<td>RM14407</td>
<td>3.8</td>
<td>−19.0</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>RM22459</td>
<td>5.2</td>
<td>−7.8</td>
<td>13.0</td>
</tr>
<tr>
<td>2008</td>
<td>BFOT</td>
<td>3</td>
<td>RM14407</td>
<td>8.4</td>
<td>−23.7</td>
<td>19.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>RM19715</td>
<td>2.5</td>
<td>4.3</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>RM22459</td>
<td>5.6</td>
<td>−17.7</td>
<td>12.9</td>
</tr>
</tbody>
</table>

$^a$ Additive effect of the EMF20 allele.

$^b$ Variance explained (%).

### FOT of NILs carrying qEMF3

Segregation analysis for BFOT and PFOT was performed by using BC$_3$F$_2$ plants that were either EMF20 homozygous, Nanjing 11 homozygous, or heterozygous in the qEMF3 region (Fig. 2A). The BFOT of EMF20-homozygous plants (06.54) was significantly earlier than that of Nanjing 11 (08.23; i.e. an 89-min difference), and also earlier than that of heterozygous plants (07.47), although the latter difference did not reach statistical significance (Fig. 2B). The BFOTs of Nanjing 11-homozygous plants (08.30) and Nanjing 11 were similar. The PFOTs of the EMF20-homozygous (08.50), heterozygous (09.21), Nanjing 11-homozygous (10.20), and Nanjing 11 (10.24) followed a pattern similar to that of BFOT (Fig. 2C). The results confirmed that a single QTL for EMF, qEMF3, significantly advanced the FOT of Nanjing 11 by ~1.5 h.

### Nanjing 11 vs Nanjing 11+qEMF3: elevated temperature experiment in a greenhouse and heat tolerance test

To prove the effectiveness of qEMF3 in escaping heat stress at flowering, the NIL developed (Fig. 3A) was subjected to the elevated high temperature from early morning to noon in the greenhouse. In this experiment, air temperature steadily increased from ~28.0°C to ~34.0°C by 10.00, then exceeded 35.0°C, which is generally the threshold for induction of spikelet sterility (Satake and Yoshida, 1978; Matsui et al., 1997), and reached 38.0–40.0°C at 11.00–15.00 (Table 2). The relative humidity steadily decreased (from >80.0% at 06.00 to ~40.0% by 15.00; Table 2). Nanjing 11+qEMF3 clearly showed peak flowering at 07.00–08.00 and completed flowering by 11.00, whereas Nanjing 11 started flowering after 07.00, showed two peaks (09.00–10.00 and 11.00–12.00), and finished flowering by 14.00 (Fig. 3B). After 10.00, only 4.1% of spikelets in Nanjing 11+qEMF3 flowered, whereas 43.1% of spikelets flowered in Nanjing 11. The percentage of sterile spikelets was significantly lower in Nanjing 11+qEMF3 (1.2%) than in Nanjing 11 (49.8%) (Fig. 3C). In a chamber experiment for heat tolerance set constantly at 38°C for 6 h on the day of flowering, spikelet sterility was similar between Nanjing 11 (33.9%) and Nanjing 11+qEMF3 (30.3%) (Fig. 3D). Thus, Nanjing 11+qEMF3 escaped heat-induced spikelet sterility by flowering earlier in the morning (i.e. at cooler temperatures).

### IR64 vs IR64+qEMF3: flowering patterns at elevated high temperatures

We also transferred the EMF20 allele of qEMF3 into IR64 through marker-assisted selection (Supplementary Figure S1B).
The graphical genotype of the NIL developed (IR64+qEMF3) is shown in Fig. 4A. Under field conditions at IRRI, mean T50 of IR64+qEMF3 was 1.9 h in the wet season and 2.2 h in the dry season (Fig. 4B), also confirming the effectiveness of qEMF3 in the genetic background of IR64. These T50s were significantly earlier (by ~2 h) than those of IR64. The IR64+qEMF3 plants were further tested in the environmentally controlled chamber to understand the response of FOT to the elevated temperature. In the first temperature regime, T10 of IR64 and IR64+qEMF3 was ~11.00 and ~09.30, respectively, T50 was ~11.30 and ~10.30, and T90 was ~12.30 and ~11.00 (Fig. 5A). Temperature at T90 was ~35.5°C (IR64) and ~33.0°C (IR64+qEMF3). Spikelet opening started
within 30 min after light exposure in IR64+qEMF3. In the third temperature regime, which was the most severe among the three regimes, T10 of IR64 and IR64+qEMF3 was ~10.30 and ~07.30, respectively, T50 was ~11.00 and ~09.00, and T90 was ~12.00 and ~10.00 (Fig. 5C). Temperature at T90 was ~38.5°C (IR64) and ~34.0°C (IR64+qEMF3). Spikelet sterility in the third temperature regime was significantly lower in IR64+qEMF3 (11.2%) than in IR64 (55.8%) (Fig. 5D). Notably, the temperature at T90 for IR64+qEMF3 was below the sterility threshold (35°C) in all three temperature regimes. In the heat tolerance test constantly at
38°C for 6 h on the day of flowering, sterility was similar in IR64 (50.2%) and IR64+qEMF3 (57.6%) (Fig. 5E).

Comparison of FOT between Nanjing 11+qEMF3 and popular local cultivars in the tropics and subtropics

The mean T50 ranged from 3.4 to 4.8 h after dawn (HAD) among popular cultivars (Table 3) in the dry season (Fig. 6). Swarna, an Indian cultivar, had the earliest T50 among the tropical and subtropical cultivars. Nanjing 11 and IR64 were placed in the early-T50 group. Caiapo, an upland cultivar from Latin America, had the latest T50 (3 h later than Nanjing 11+qEMF3). The mean T50 of Nanjing 11+qEMF3 was 1.8 HAD, which was significantly earlier than those of all tested cultivars (Fig. 6). The difference between T10 and T90 varied among the accessions: the difference was >1.5 h for Nanjing 11, TDK1, KDML105, Epagri108, Pusa Basmati, and Caiapo, and was less for Ciherang and Sahel 329. For Nanjing 11+qEMF3, the mean T10 was 1.1 HAD and the mean T90 was 2.5 HAD; this T90 was earlier than T10 of all other cultivars. The mean T10 was 1.1 HAD and the mean T90 was 2.5 HAD; this T90 was earlier than T10 of all other cultivars. This result indicates that Nanjing 11+qEMF3 had almost completed flowering prior to the beginning of FOT of the tested popular cultivars. The T50 of CG14 was 40 min to 2 h earlier than those of other cultivars but significantly later than that of Nanjing 11+qEMF3; T90 of Nanjing 11+qEMF3 was similar to T10 of CG14 (Fig. 6).

Discussion

We attempted to develop NILs for EMF in indica-type genetic backgrounds through the detection of QTLs using a wild rice, *O. officinalis*, as a donor parent, aiming for genetic improvement of FOT to the cooler early morning. Heat-induced spikelet sterility is expected to aggravate rice yield losses in vulnerable temperate, subtropical, and tropical regions. We also investigated the FOTs of cultivars popular in tropical and subtropical rice-growing regions to identify the presence or absence of the EMF trait for the broader application of QTLs detected to rice breeding.

qEMF3 contributes to heat escape at flowering by advancing FOT to early morning

To date, there has been only one attempt to detect QTLs for EMF derived from *O. rufipogon* (AA genome; Thanh et al., 2010); this study detected QTLs for BFOT on chromosomes 5 and 12, and QTLs for PFOT on chromosomes 4 and 5. However, the effects of these QTLs have not been

Table 3. Cultivars and lines used in this study

<table>
<thead>
<tr>
<th>Name of cultivar or line</th>
<th>Accession no.</th>
<th>Cultivar group</th>
<th>Area</th>
<th>Main countries of cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanjing11+qEMF3</td>
<td>IRIS251–49436</td>
<td>Indica-type</td>
<td>East Asia</td>
<td>China</td>
</tr>
<tr>
<td>Nanjing11</td>
<td>IRIS251–49437</td>
<td>Indica-type</td>
<td>East Asia</td>
<td>Bhutan, Burkina Faso (FKR42), Cambodia, Ecuador (INIA11), Gambia, India, Indonesia, Mauritania</td>
</tr>
<tr>
<td>IR64</td>
<td>IRGC66970</td>
<td>Indica-type</td>
<td>Southeast Asia</td>
<td></td>
</tr>
<tr>
<td>NSIC Ric222</td>
<td>IRTF 24370</td>
<td>Indica-type</td>
<td>Southeast Asia</td>
<td>The Philippines</td>
</tr>
<tr>
<td>Cihang</td>
<td>IRTF 25143</td>
<td>Indica-type</td>
<td>Southeast Asia</td>
<td>Indonesia</td>
</tr>
<tr>
<td>KDML 105</td>
<td>IRTF 6886</td>
<td>Indica-type</td>
<td>Southeast Asia</td>
<td>Thailand</td>
</tr>
<tr>
<td>TDK1</td>
<td>IRIS 1–3354</td>
<td>Indica-type</td>
<td>Southeast Asia</td>
<td>Laos</td>
</tr>
<tr>
<td>BR11</td>
<td>IRIS 109–4361</td>
<td>Indica-type</td>
<td>South Asia</td>
<td>Bangladesh</td>
</tr>
<tr>
<td>MTU1010</td>
<td>IRTF 23154</td>
<td>Indica-type</td>
<td>South Asia</td>
<td>India</td>
</tr>
<tr>
<td>Pusa Basmati</td>
<td>IRIS 10–90048</td>
<td>Indica-type</td>
<td>South Asia</td>
<td>India</td>
</tr>
<tr>
<td>Swarna</td>
<td>IRTF 12715</td>
<td>Indica-type</td>
<td>South Asia</td>
<td>India</td>
</tr>
<tr>
<td>ADT36</td>
<td>IRGC 64818</td>
<td>Indica-type</td>
<td>South Asia</td>
<td>India</td>
</tr>
<tr>
<td>Sambhia Mahsuri</td>
<td>IRTF 24472</td>
<td>Indica-type</td>
<td>South Asia</td>
<td>India</td>
</tr>
<tr>
<td>BG90-2</td>
<td>IRGC 116968</td>
<td>Indica-type</td>
<td>Africa</td>
<td>Mali, Gambia, Ethiopia, Nigeria</td>
</tr>
<tr>
<td>Bouake 189</td>
<td>IRGC 78169</td>
<td>Indica-type</td>
<td>Africa</td>
<td>Ivory Coast, Guinea</td>
</tr>
<tr>
<td>Moroberekan</td>
<td>IRGC 12048</td>
<td>Japonica-type</td>
<td>Africa</td>
<td>Ivory Coast</td>
</tr>
<tr>
<td>Nerica L-19</td>
<td>IRIS 253–895327</td>
<td>Indica-type</td>
<td>Africa</td>
<td>Nigeria, Mali, Burkina Faso, Liberia, Sierra Leone, Cameroon, Togo</td>
</tr>
<tr>
<td>Sahel 108</td>
<td>IRIS 251–34260</td>
<td>Indica-type</td>
<td>Africa</td>
<td>Senegal, Mauritania</td>
</tr>
<tr>
<td>Sahel 134</td>
<td>WAB 15822</td>
<td>Indica-type</td>
<td>Africa</td>
<td>Senegal, Mauritania</td>
</tr>
<tr>
<td>Sahel 329</td>
<td>WAS 197–B-4–1–5</td>
<td>Indica-type</td>
<td>Africa</td>
<td>Senegal, Mauritania</td>
</tr>
<tr>
<td>BR-Img 410</td>
<td>IRIS 294–6196</td>
<td>Indica-type</td>
<td>Latin America</td>
<td>Southern Brazil</td>
</tr>
<tr>
<td>Caiapo</td>
<td>IRIS 294–6354</td>
<td>Indica-type</td>
<td>Latin America</td>
<td>Northern Brazil</td>
</tr>
<tr>
<td>Epagri 108</td>
<td>IRIS 294–6315</td>
<td>Indica-type</td>
<td>Latin America</td>
<td>Southern Brazil, Venezuela</td>
</tr>
<tr>
<td>Fedebrmoz 50</td>
<td>IRIS 298–9831</td>
<td>Indica × japonica</td>
<td>Latin America</td>
<td>Colombia, Venezuela, Costa Rica, Panama</td>
</tr>
<tr>
<td>Oryzica 1</td>
<td>IRIS 294–5912</td>
<td>Indica-type</td>
<td>Latin America</td>
<td>Colombia</td>
</tr>
<tr>
<td>CG14</td>
<td>IRGC 96717</td>
<td>O. glaberrima</td>
<td>Africa</td>
<td></td>
</tr>
</tbody>
</table>

The flowering patterns of CG14 were observed in 2013.
functionally validated through the development of NILs, which makes it difficult to use these QTLs in breeding programmes. In our study, QTLs for BFOT and PFOT derived from *O. officinalis* were found on chromosomes 3 (qEMF3) and 8 (qEMF8) (Table 1); thus, we consider them to be different from those identified by Thanh et al. (2010). We confirmed the allelic effect of EMF20 on qEMF3 for EMF in the BC1F2 population (Fig. 2B, C) and developed NILs for EMF in the genetic background of Nanjing 11 (Fig. 3A) and IR64 (Fig. 4A). Comparison of FOT between the recurrent parents and the NILs revealed that the EMF20 allele of qEMF3 advanced FOT by 1.5–2.0 h both in temperate Japan (Fig. 2B, C) and the tropical Philippines across different seasons (Fig. 4B), indicating that qEMF3 advances the FOT under various environmental conditions.

Our previous study revealed that EMF20, an introgression line containing both qEMF3 and qEMF8, avoids heat-induced spikelet sterility at flowering under an elevated temperature in the greenhouse (Ishimaru et al., 2010). In this study, we found two QTLs for the EMF trait, qEMF3 and qEMF8 (Table 1). We tested the hypothesis that a single QTL, qEMF3, is effective in avoiding heat stress at flowering using Nanjing 11+qEMF3 and IR64+qEMF3. By 10.00, when the temperature exceeded 35°C (Table 2), over 95% of spikelets had completed flowering in Nanjing 11+qEMF3 but only 57% in Nanjing 11 (Fig. 3B). As a result, spikelet sterility was significantly reduced in Nanjing 11+qEMF3 (Fig. 3C), although sterility was similar in Nanjing 11+qEMF3 and Nanjing 11 in the heat tolerance test (Fig. 3D). This result supports our hypothesis that qEMF3 is sufficient to advance the FOT of Nanjing 11, which allows this cultivar to escape heat stress during the daytime. Using an environmentally controlled chamber and three different temperature regimes, we confirmed that almost all spikelets of IR64+qEMF3 flowered before the temperature reached 35°C, whereas the percentage of spikelets that opened at temperatures below 35°C varied in IR64 depending on the temperature regime (Fig. 5A–C). This difference in the flowering pattern significantly reduced spikelet sterility in IR64+qEMF3 (Fig. 6D). The results clearly show that qEMF3 has a similar effect on FOT in the IR64 background. Thus, qEMF3 contributes to heat escape at flowering by advancing FOT. It is notable that qEMF3 shifted its FOT drastically responding to the given temperature regimes (Fig. 5A–C). The NILs developed could be a novel material for revealing the interaction of genetic and environmental control of FOT.

**qEMF3 has the potential to shift the FOT of cultivars popular in the tropics and subtropics to earlier in the morning.**

The vulnerability of the major rice-producing regions to heat-induced spikelet sterility at the flowering stage has been mapped both regionally (Wassmann et al., 2009) and globally (Teixeira et al., 2013). Although major local cultivars listed in Table 3 occupy a large proportion of rice cultivation areas in the tropics and subtropics, no assessment of their FOT traits has been conducted so far. Our investigation of the flowering patterns revealed that none of them had the EMF trait (Fig. 6). Among them, Nanjing 11 and IR64 were categorized in the early-FOT group (Fig. 6). The advancement in FOT due to qEMF3 in the genetic background of Nanjing 11 (Fig. 2B, 2C; Fig. 7) and IR64 (Fig. 4B) shows that qEMF3 has a significant impact even on the early-FOT cultivars.

Heat-induced spikelet sterility occurs even upon exposure of a flowering spikelet to a short-term heat stress (1 h at ~37°C; Jagadish et al., 2007). However, similar heat exposure 1 h after flowering hardly affects fertility (Satake and Yoshida, 1978; Ishimaru et al., 2010). Therefore, advancing FOT by 1 h results in a significant difference in the proportion of sterile spikelets in rice under heat stress. Among the cultivars tested, Caiapo had the latest FOT, 3 h later than Nanjing 11+qEMF3 (Fig. 6). Two-thirds of the cultivars tested had their T50 2 h later than Nanjing 11+qEMF3 (Fig. 6), and these cultivars should be prioritized as targets for breeding programmes for EMF through the introgression of qEMF3. The fact that none of the popular cultivars tested, including CG14, which is known as an EMF cultivar (Jagadish et al., 2008), had the same EMF trait as Nanjing 11+qEMF3 shows a pressing need to transfer qEMF3 to those cultivars by marker-assisted breeding. The allele of the EMF20 locus from *O. officinalis* has a potential to shift the FOT of these cultivars to a time earlier in the morning when the temperature is lower. Thus, the wild rice, *O. officinalis*, could provide an excellent genetic resource for the EMF trait to mitigate heat-induced spikelet sterility at flowering, which would help in breeding rice cultivars able to cope with future hotter climates.

**Supplementary Data**

**Supplementary Table 1.** Meteorological data inside the greenhouse at IRRI in the 2012 and 2013 dry season.
Supplementary Figure S1. Breeding scheme for the development of NILs of rice (O. sativa) with the cultivars Nanjing 11 and IR64.

Supplementary Figure S2. An example of the T10, T50, and T90 calculation for three cultivars on a single day (22 February 2012).

Funding
This study was supported by the Ministry of Foreign Affairs and the Ministry of Agriculture, Forestry, and Fisheries of Japan to T.I. and N.K., Federal Ministry for Economic Cooperation and Development, Germany to K.S.V.J., and the National Agricultural and Food Research Organization (NARO), Japan to T.I. and H.H.

Acknowledgements
Our thanks go to Dr T. Imbe (NARO), Dr M. Iwanaga (JIRCAS), Dr D.S. Brar and Dr E. Nissilä and Dr A. Dorbermann (IRRI) for their encouragement. Our thanks also go to Dr R. Muthurajan (Tamil Nadu Agricultural University), Dr B. Mameh (Africa Rice), and Dr E. Torres (CIAT) for providing information on popular local cultivars.

References


IPCC. 2013. Fifth Assessment Report (AR5) : WMO/UNEP


