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Differential contribution of two Ppd-1 homoeoalleles to early-flowering phenotype in Nepalese and Japanese varieties of common wheat

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Wheat landraces carry abundant genetic variation in heading and flowering times. Here, we studied flowering-related traits of two Nepalese varieties, KU-4770 and KU-180 and a Japanese wheat cultivar, Shirogane-komugi (SGK). These three wheat varieties showed similar flowering time in a common garden experiment. In total, five significant quantitative trait loci (QTLs) for three examined traits, the heading, flowering and maturation times, were detected using an F₂ population of SGK/KU-4770. The QTLs were found at the Ppd-1 loci on chromosomes 2B and 2D and the 2B QTL was also confirmed in another F₂ population of SGK/KU-180. The Ppd-D1 allele from SGK and the Ppd-B1 alleles from the two Nepalese varieties might be causal for early-flowering phenotype. The SGK Ppd-D1 allele contained a 2-kb deletion in the 5′ upstream region, indicating a photoperiod-insensitive Ppd-D1a allele. Real-time PCR analysis estimating the Ppd-B1 copy number revealed that the two Nepalese varieties included two intact Ppd-B1 copies, putatively resulting in photoperiod insensitivity and an early-flowering phenotype. The two photoperiod-insensitive Ppd-1 homoeoalleles could independently contribute to segregation of early-flowering individuals in the two F₂ populations. Therefore, wheat landraces are genetic resources for discovery of alleles useful for improving wheat heading or flowering times.

Key Words: copy number variation, flowering time, landraces, photoperiodic sensitivity, Triticum aestivum L.

Introduction

Common wheat cultivars show widely varying heading times (HT) in order to adapt to different regional environments (Snape et al. 2001). Heading/flowering time (HT/FLT) is one of the important traits in wheat breeding. Wheat HT/FLT is controlled by three major genetic components, vernalization requirement, photoperiodic sensitivity and narrow-sense earliness (earliness per se) (reviewed in Murai et al. 2005). A few major genes control the vernalization requirement and determine spring and winter habits of each wheat cultivar. The photoperiodic sensitivity is mainly determined by the homoeologous loci Ppd-A1, Ppd-B1 and Ppd-D1, which are located on the short arms of chromosomes 2A, 2B and 2D, respectively (Law et al. 1978, Scarth and Law 1983, Welsh et al. 1973). Narrow-sense earliness is the earliness of fully vernalized plants grown under long days, and no major genes have been detected for this character (Murai et al. 2005). Nevertheless, the earliness per se genes are known to be located on several chromosomes including group 2 homoeologous chromosomes (Scarth and Law 1983). It has been reported that some chromosomes include quantitative trait loci (QTLs) controlling narrow-sense earliness (Hanoq et al. 2004, Hoogendoorn 1985, Law 1987, Lin et al. 2008, Miura and Worland 1994).

Most spring type landraces of southwest Japan carry a dominant Vrn-D1 allele (Gotoh 1979, Iwaki et al. 2000, Seki et al. 2011). The spring habit caused by the Vrn-D1 allele shows slightly short narrow-sense earliness (Kato et al. 2001). It is significant that photoperiodic sensitivity is an adaptive trait in common wheat cultivars (Worland et al. 1998). A Ppd-D1a mutant allele is photoperiod insensitive because of a 2-kb deletion upstream of the coding region in a pseudo-response regulator gene on wheat chromosome 2D (Beales et al. 2007, Nishida et al. 2013). The photoperiod-insensitive allele has been widely distributed for breeding of early-flowering cultivars, and was originally derived from the Japanese wheat variety Akakomugi (Borojevic and Borojevic 2005, Worland et al. 1998). Recently, haplotype analysis of the Ppd-D1 gene implied that the Ppd-D1a allele might have originated from a photoperiod-sensitive Ppd-D1b allele in Asia (Guo et al. 2010). Most Japanese wheat cultivars, except those in the Hokkaido area, carry the Ppd-D1a allele, which contributes to their early-flowering phenotype, helping to avoid damage from preharvest rain (Seki et al. 2011). Moreover, structural variations have been found...
at the Ppd-B1 locus (Beales et al. 2007, Nishida et al. 2013, Takenaka and Kawahara 2012). It was recently reported that the copy number of Ppd-B1 is associated with FLT variation in common wheat (Diaz et al. 2012) and that structural variation exists between the 5′ upstream region of the photoperiod-insensitive Ppd-B1a allele and the photoperiod-sensitive Ppd-B1b allele (Nishida et al. 2013).

Wheat landraces provide abundant genetic variation in many agricultural traits including FLT (Iwaki et al. 2001, Kato and Yokoyama 1992, Terasawa et al. 2009) and some landraces supply useful traits for modern wheat breeding (Feldman and Sears 1981). In previous studies, we found that many wheat landraces in Nepal, Bhutan and Tibet show wide variation in flowering traits (Kato and Yokoyama 1992, Takumi 2009). A common garden experiment revealed the presence of early-flowering landraces in Nepal, which have FLT as short as that of the Japanese early-flowering cultivar Shiroganekomugi (SGK) (Takumi 2009). HT is highly correlated with photoperiodic response and narrow-sense earliness in the Nepalese and Bhutanese landraces and in particular, narrow-sense earliness shows significant association with altitude of landrace collection sites in Nepal (Kato and Yokoyama 1992). However, there is little information about the genetic basis of flowering traits in the Nepalese and Bhutanese wheat landraces.

Early-maturing cultivars of common wheat are required in Japan, because the rainy season overlaps with that of wheat maturation. High moisture at maturation time (MAT) results in preharvest sprouting and Fusarium damage and reduction of wheat grain quality. Earliness of HT/FLT in the previously identified Nepalese landraces might be caused by different genetic loci than the early-flowering phenotype of SGK. Therefore, our objective was to identify causal loci for early flowering in the Nepalese landrace and SGK. Based on the results, genetic differences in FLT between the Nepalese and Japanese landraces and their usefulness for wheat breeding were discussed.

Materials and Methods

Plant materials

Three accessions of common wheat, including the Japanese cultivar Shiroganekomugi (SGK) and two Nepalese landraces, KU-4770 and KU-180, were used in this study. These three accessions exhibited spring growth habit, and the two Nepalese landraces were identified in a screen of 41 accessions from Nepal, Bhutan and Tibet (Kajimura et al. 2011). These three accessions exhibit spring growth habit, and the two Nepalese landraces, KU-4770 and KU-180, were used in this study.

Phenotype measurement and statistical analyses

Four flowering-related traits were measured at the field. Heading time (HT) and flowering time (FLT) were recorded as days after sowing as shown in our previous report (Kajimura et al. 2011). Maturity time (MAT) was measured as the number of days that had passed before the peduncle turned yellow, according to our previous report (Kajimura et al. 2011). The grain filling period (GFP) was the number of days from flowering to maturation. HT, FLT and MAT were measured for the three earliest tillers of each plant and mean values were calculated using the data for each F2 plant. The data were statistically analyzed using JMP software ver. 5.1.2 (SAS Institute, Cary, NC, USA). Pearson’s correlation coefficients were estimated among the traits measured in each mapping population.

Detection of polymorphisms and genotyping with molecular markers

To amplify PCR fragments of simple sequence repeat (SSR) markers, total DNA was extracted from the parents and F2 individuals using standard procedures. For SSR genotyping, 40 cycles of PCR were performed using 2x Quick Taq HS DyeMix (TOYOBO, Osaka, Japan) with the following conditions: 10 s at 94°C, 30 s at the annealing temperature and 30 s at 68°C. The last step was incubation for 1 min at 68°C. Information on the SSR markers and their annealing temperatures was obtained from the National BioResource Project (NBRP) KOMUGI web site (http://www.shigen.nig.ac.jp/wheat/komugi/strains/aboutNbrpMarker.jsp) and the GrainGenes web site (http://wheat.pw.usda.gov/GG2/maps.shtml). The PCR products were separated in 2% agarose or 13% nondenaturing polyacrylamide gels and visualized under UV light after staining with ethidium bromide. For polyacrylamide gel electrophoresis, the high efficiency genome scanning system (Nippon Eido, Tokyo, Japan) of Hori et al. (2003) was used.

Polymorphism at the Ppd-D1 locus was detected using allele-specific primers according to Beales et al. (2007). A common forward primer, Ppd-D1_F, 5′-ACGCCTCCAC TACACTG-3′ and two reverse primers, Ppd-D1_R1, 5′-GGTTGTTCACAACAGAGC-3′ and Ppd-D1_R2, 5′-CACTGGTGTTAGCAGATT-3′, were used for this PCR-based analysis. PCR products amplified with Ppd-D1_F and Ppd-D1_R2 showed a 2,089-bp deletion in the 5′ upstream region of Ppd-D1, indicative of the photoperiod-insensitive Ppd-D1a allele (Beales et al. 2007). Ppd-B1 alleles were determined using the following allele-specific primers for amplification by PCR: Ppd-B1_2ndcopy_F1, 5′-TAACGTCTGCTCAAAAGTG-3′ and Ppd-B1_2ndcopy_R1, 5′-CCGGAACTGAGGATCATC-3′ (Beales et al. 2007). PCR products amplified with Ppd-B1_2ndcopy_F1
and Ppd-B1_2ndcopy_R1 gave a 425 bp fragment derived from the partly deleted second Ppd-B1 copy in CS (Beales et al. 2007). The PCR products were separated by electrophoresis through a 1.2% agarose gel and stained with ethidium bromide.

**Map construction and QTL analysis**

The polymorphic SSRs, Ppd-B1 and Ppd-D1 of the parents were genotyped and used for map construction. Genetic mapping was performed using MAPMAKER/EXP software ver. 3.0 (Lander et al. 1987). The threshold value for log-likelihood (LOD) scores was set at 3.0 and the genetic distances were calculated using the Kosambi mapping function (Kosambi 1944). Chromosomal assignment of SSR markers was generally based on reported reference maps (Gupta et al. 2002, Kobayashi et al. 2010, Pestsova et al. 2000, Röder et al. 1998, Somers et al. 2004, Torada et al. 2006).

QTL analyses were carried out by composite interval mapping using Windows QTL Cartographer software ver. 2.5 (Wang et al. 2011) using the forward and backward method. A LOD score threshold for each trait was determined by computing a 1,000-permutation test. The percentage of phenotypic variation explained by a QTL for a trait and any additive effects were also estimated using this software.

**Copy number estimation of Ppd-B1**

Real-time PCR analysis was carried out for estimation of copy number of the Ppd-B1 genes using a LightCycler 480 Real-Time PCR System II (Roche Diagnostics, Basel, Switzerland). The wheat CONSTANS2 gene, TaCO2, was used as an internal control. Gene-specific primer sets were based on a previous report (Díaz et al. 2012): 5′-GCCGTAAGTTA CTATCTCTCATGTTATC-3′ and 5′-TTGTTTTTAGT ACCCAGTACCATACACG-3′ for Ppd-B1 and 5′-TGCTA ACCGTTGGCATCAC-3′ and 5′-GGTACATAGTGCTG CTGCGATCTG′-3′ for TaCO2. The rate of amplification was monitored using THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan) according to the manufacturer’s protocol. The relative copy number of Ppd-B1 was calculated as 2-ΔΔCt, where ΔCt is the difference in number of PCR cycles required to reach the log phase of amplification of Ppd-B1 relative to TaCO2; representative values were expressed relative to the Ppd-B1 copy number in the Cmn genome.

**Assay for flowering-related traits under short-day conditions**

For estimation of photoperiod requirement, five imbibed seeds of each wheat cultivar were planted in a pot with soil and incubated for two weeks at 24°C with a 12 h light/12 h dark photoperiod at a light intensity of 55–65 μmol m-2 s-1 provided by cool white fluorescent lamps. The seedlings were treated at 4°C for one week for vernalization and then transferred to 24°C with a 12 h photoperiod. The four flowering-related traits, HT, FLT, MAT and GFP, were recorded for each cultivar.

**Results**

**Flowering-related traits in two F2 populations and their parental accessions**

The mean values of two Nepalese varieties, KU-4770 and KU-180, were compared with SGK. HT of the Nepalese varieties was 4 to 8 days later than SGK, whereas there were no significant differences for FLT, MAT and GFP (Table 1). Little variation in the four traits was observed among individual plants of each parental variety. However, all four traits varied widely in the F2 populations. The SGK/KU-180 population showed larger variance in GFP than the SGK/ KU-4770 population compared with the variance in the other three traits. Much earlier and later heading/flowering F2 plants were present than in the parental lines, which indicated transgressive segregation. These observations implied that genes controlling the early heading/flowering of the Nepalese varieties may be distinct from those of SGK.

Significant (P < 0.001) positive correlations were observed among HT, FLT and MAT in the two F2 populations (Table 2). MAT correlated significantly (P < 0.001) with GFP, while HT and FLT did not show any positive correlation with GFP in the two F2 populations. In the SGK/KU-

**Table 1. Parental and F2 population means for four flowering-related traits measured in each of the two F2 mapping populations**

| Population          | Heading time | Flowering time | Maturation time | Grain-filling period |
|---------------------|--------------|----------------|-----------------|----------------------|
| SGK/KU-4770 in 2008–2009 season |              |                |                 |                      |
| SGK                 | 130.3 ± 1.18* | 148.0 ± 0.47 | 179.8 ± 0.88    | 31.8 ± 0.57         |
| KU-4770             | 138.9 ± 0.83 | 149.9 ± 0.50  | 180.1 ± 0.91    | 30.1 ± 0.57         |
| F1 plants           | 138.1 ± 0.87 | 150.0 ± 0.59  | 180.0 ± 1.10    | 30.0 ± 0.71         |
| Mean in F1 plants   | 136.0        | 149.9         | 180.6           | 30.7                |
| Range in F1 plants  | 124.0–153.0  | 143.0–161.0   | 168.3–193.6     | 19.6–36.6           |
| Variance in F1 plants | 43.96      | 11.51         | 16.41           | 4.99                |
| SGK/KU-180 in 2009–2010 season |              |                |                 |                      |
| SGK                 | 132.6 ± 0.89 | 147.0 ± 1.00  | 181.6 ± 1.14    | 34.6 ± 1.89         |
| KU-180              | 136.0 ± 1.22 | 147.8 ± 0.45  | 179.8 ± 0.83    | 32.0 ± 0.70         |
| Mean in F2 plants   | 133.1        | 145.8         | 183.2           | 37.4                |
| Range in F2 plants  | 123.3–157.0  | 137.6–163.0   | 174.6–196.5     | 27.33–49.7          |
| Variance in F2 plants | 33.66      | 25.33         | 21.83           | 22.30               |

*Means (days) with standard deviations.

**Table 2. Correlation coefficient (r) matrices for four traits measured in two F2 populations**

| Population          | Heading time | Flowering time | Maturation time |
|---------------------|--------------|----------------|----------------|
| SGK/KU-4770 in 2008–2009 season |              |                |                 |
| Flowering time      | 0.9414***    | 0.7991***      | 0.8278***       |
| Maturation time     | 0.9414***    | 0.7991***      | 0.8278***       |
| Grain-filling period | 0.0129      | −0.0112        | 0.5375***       |
| SGK/KU-180 in 2009–2010 season |              |                |                 |
| Flowering time      | 0.9704***    | 0.5181***      | 0.5441***       |
| Maturation time     | 0.9704***    | 0.5181***      | 0.5441***       |
| Grain-filling period | −0.5439***  | −0.5496***     | 0.3979***       |

Levels of significance are indicated by asterisks, ***P < 0.001.
180 population, GFP was negatively correlated with HT and FLT. The negative correlation between GFP and HT/FLT indicated that the earlier flowering $F_2$ individuals required a longer period for grain maturation and that GFP in the late flowering $F_2$ individuals tended to be shorter.

**Construction of a linkage map and QTL analysis for flowering-related traits in the SGK/KU-4770 population**

In the SGK/KU-4770 population, 889 primer sets were tested, including 887 SSR primer sets and $Ppd-D1$ and $Ppd-B1$ allele-specific primers and 166 (16.7%) were found to be polymorphic between the parental accessions. Of these, 145 SSR markers, $Ppd-D1$ and $Ppd-B1$ formed 33 linkage groups. The total map length was 1,649 cM with an average spacing of 11.4 cM between markers.

QTLs for the three flowering-related traits other than GFP were detected using the SGK/KU-4770 genetic map. In total, 5 QTLs, located on chromosomes 2B and 2D, showed significant LOD scores ($P < 0.05$) (Fig. 1). Two, one and two QTLs were respectively detected for HT, FLT and MAT (Table 3).

For HT, two QTLs were detected on the short arms of chromosomes 2B and 2D with LOD scores of 5.26 and 18.84, respectively (Table 3). The two QTLs, located at the $Ppd-B1$ and $Ppd-D1$ chromosomal regions, respectively explained 8.8% and 58.0% of the variation in HT in the SGK/KU-4770 population. The additive effect of the HT QTLs indicated that the KU-4770 allele at the 2B QTL promoted heading earlier than the SGK allele, while the SGK allele at the 2D QTL has an earlier heading effect than the KU-4770 allele.

For FLT, one QTL with a LOD score of 15.39 was found on chromosome 2D. The 2D QTL for FLT was located at the $Ppd-D1$ chromosomal position similar to that of HT and contributed 50.7% of the FLT variation. The additive effect of the FLT QTL on chromosome 2D showed the same direction as that of the 2D QTL for HT.

For MAT, two QTLs with LOD scores of 6.36 and 11.66 were respectively found in the $Ppd-B1$ and $Ppd-D1$ chromosomal regions. The 2B and 2D QTLs explained 9.1% and 37.2%, respectively, of the variation in MAT. The SGK allele at the MAT QTL on 2D had an earlier effect on MAT than the KU-4770 allele, whereas the KU-4770 allele at the MAT QTL on 2B contributed to early maturation.

To study the effects of the identified QTLs, data on each of the flowering-related traits, HT, FLT and MAT, were grouped based on the genotypes at the QTL regions of each $F_2$ individual. For all three traits, there were significant ($P < 0.05$) differences among genotypes at the QTLs.

![Fig. 1. Linkage maps and positions of QTLs identified on the short arms of chromosomes 2B and 2D for three flowering-related traits. QTLs with LOD scores above the threshold are indicated and genetic distances (in centimorgans) are given to the right of each chromosome. Black arrowheads indicate the putative positions of centromeres.](image-url)
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The $F_2$ individuals homozygous for the KU-4770 allele at $Ppd-B_1$ showed significantly earlier HT, FLT and MAT than those homozygous for the SGK allele. Conversely, the $F_2$ individuals homozygous for the SGK allele at $Ppd-D_1$ exhibited earlier attributes than those homozygous for the KU-4770 allele.

**Confirmation of effects of the identified QTLs in the SGK/KU-180 population**

To confirm the expression of the 2B QTL identified in the SGK/KU-4770 population, another $F_2$ population of SGK/KU-180 was examined. A linkage map for the short arm of chromosome 2B was constructed for the SGK/KU-180 population. No polymorphism was detected using the $Ppd-B_1$_2ndcopy_F1 and $Ppd-B_1$_2ndcopy_R1 primers for $Ppd-B_1$, but in total, 9 SSR markers could be mapped in 2BS. QTL analyses for the four flowering-related traits were conducted using the linkage map and three QTLs for HT, FLT and MAT with respective LOD scores of 3.44, 3.46 and 3.76 were found on chromosome 2B in the SGK/KU-180 population (Fig. 1). The 2B QTLs were located between $Xwmc770$ and $Xbarc13$ and the chromosomal positions corresponded in the two $F_2$ populations. These QTLs contributed 18–30% of the variation in HT, FLT and MAT in the SGK/KU-180 population (Table 3). In addition, additive effects indicated that the KU-180 alleles at the 2B QTLs show earlier attributes than the SGK alleles.

**Polymorphisms between SGK and KU-180**

Table 3. A summary of QTLs for flowering-related traits that were identified in the two $F_2$ mapping populations

| Traits | Chromosome | Map location          | LOD score | LOD threshold | Contribution (%) | Additive effect |
|--------|------------|-----------------------|-----------|---------------|-----------------|----------------|
| SGK/KU-4770 |            |                       |           |               |                 |                |
| HT     | 2B         | $Ppd-B_1$-Xbarc13     | 5.26      | 4.9           | 8.84            | −2.63          |
| HT     | 2D         | $Ppd-D_1$-Xgwm484     | 18.84     | 4.9           | 57.98           | 6.34           |
| FLT    | 2D         | $Ppd-D_1$-Xgwm484     | 15.39     | 4.5           | 50.73           | 4.01           |
| MAT    | 2B         | $Ppd-B_1$-Xbarc13     | 6.36      | 6.2           | 9.09            | −1.62          |
| MAT    | 2D         | $Ppd-D_1$-Xgwm484     | 11.66     | 6.2           | 37.17           | 3.91           |
| SGK/KU-180 |            |                       |           |               |                 |                |
| HT     | 2B         | $Xwmc770$-Xbarc13     | 3.44      | 2.4           | 18.51           | −3.43          |
| FLT    | 2B         | $Xwmc770$-Xgwm484     | 3.46      | 2.3           | 20.85           | −3.45          |
| MAT    | 2B         | $Xwmc770$-Xbarc13     | 3.76      | 2.4           | 30.41           | −3.66          |

(Fig. 2). The $F_2$ individuals homozygous for the KU-4770 allele at $Ppd-B_1$ showed significantly earlier HT, FLT and MAT than those homozygous for the SGK allele. Conversely, the $F_2$ individuals homozygous for the SGK allele at $Ppd-D_1$ exhibited earlier attributes than those homozygous for the KU-4770 allele.
Effect of Ppd-B1 of Nepalese landraces on wheat flowering traits

Each flowering-related trait was grouped based on the genotypes at the QTL regions of each F₂ individual in the SGK/KU-180 population. For HT, FLT and MAT, there were significant (P < 0.05) differences among genotypes at the 2B QTLs and Ppd-D1 (Fig. 3). The F₂ individuals homozygous for the KU-180 allele at the 2B QTL showed significantly earlier HT, FLT and MAT than those homozygous for the SGK allele. Conversely, the F₂ individuals homozygous for the SGK allele at Ppd-D1 exhibited earlier attributes than those homozygous for the KU-180 allele. The opposite effects of SGK and KU-180 alleles corresponded to those found in the SGK/KU-4770 population.

Comparison of effects of the 2B and 2D QTLs on flowering-related traits

To compare effects of the 2B and 2D QTLs on flowering-related traits, the data were grouped into four categories based on the genotypes at the QTL regions of each F₂ individual in the two populations (Fig. 4). No significant difference in the three flowering-related traits was observed among the following three groups in the two populations: F₂ individuals with only the Nepalese variety’s allele at the 2B QTL, F₂ individuals with only the SGK allele at the 2D QTL and F₂ individuals with both the Nepalese variety’s allele at the 2B QTL and the SGK allele at the 2D QTL. These three groups showed earlier HT, FLT and MAT than the other group with neither the Nepalese variety’s allele at the 2B QTL nor the SGK allele at the 2D QTL.

Copy number estimation of Ppd-B1

Although no polymorphism between SGK and KU-180 was observed using the Ppd-B1 allele-specific primer set, significant QTLs for flowering-related traits were detected in the Ppd-B1 region. It was recently reported that alleles with an increased copy number of Ppd-B1 confer an early-flowering, photoperiod-insensitive phenotype (Díaz et al. 2012). Therefore, we estimated the Ppd-B1 copy number in SGK and the two Nepalese varieties. As reported in Díaz et al. (2012), the copy number of Ppd-B1 in CS was calculated as being four times higher than in Cnn (Fig. 5). Real-time PCR analysis showed that SGK contains one copy of Ppd-B1 and KU-4770 and KU-180 respectively contain three and two Ppd-B1 copies per haploid genome. Because the 425-bp PCR fragment from the partly deleted Ppd-B1 copy was observed in KU-4770, KU-4770 was presumed to contain a truncated copy of Ppd-B1 which was previously reported (Beales et al. 2007, Díaz et al. 2012).

To examine the photoperiod insensitivity in the two Nepalese varieties, flowering-related traits were measured under short-day conditions. Both KU-4770 and KU-180 transited as well as SGK to reproductive phase under short-day conditions (Table 4), indicating that the two Nepalese varieties are photoperiod insensitive for heading and flowering. In addition, HT, FLT and MAT of KU-4770 and KU-180 were significantly earlier than the corresponding values for SGK under short-day conditions.

Fig. 3. The genotype effects at each QTL on the observed variation in the flowering-related traits in the SGK/KU-180 population. Markers that were used to deduce the genotype at a QTL are listed above each graph. The number of F₂ individuals with each genotype is indicated above the closed bars in the left graph. Means ± standard deviation with the same letter were not significantly different (P > 0.05) (Tukey-Kramer HSD test).
**Discussion**

SGK is an early-flowering Japanese cultivar of common wheat. A common garden experiment revealed that two varieties selected from Nepalese landraces showed an early flowering phenotype similar to SGK. We then performed QTL analysis to elucidate the genetic basis underlying the early FLT in these wheat varieties. Two chromosomal regions contributed large parts of the variation in HT, FLT and MAT in the two F2 populations and the two major QTL regions seem to be Ppd-B1 and Ppd-D1 (Fig. 1 and Table 3). The effects of each allele of the Ppd-D1 and Ppd-B1 on HT, FLT and MAT (Figs. 2, 3) strongly suggested that the Ppd-D1 allele from SGK and the Ppd-B1 alleles from the two Nepalese varieties caused the early-flowering phenotype in the two F2 populations. The SGK allele of Ppd-D1 contained a 2,089-bp deletion in the 5′ upstream region, indicating a photoperiod-insensitive Ppd-D1a allele (Beales et al. 2007). Most of the Japanese early-flowering cultivars, including SGK, carry the Ppd-D1a allele (Seki et al. 2011), an observation supported by the present study. On the other hand, introduction of the photoperiod-insensitive allele of Ppd-B1 into wheat cultivars has been extremely limited in Japan except in the Hokkaido region (Seki et al. 2011). The 2B QTL position identified in the present study corresponds to the
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*Ppd-B1* locus and the early attribute alleles at the 2B QTLs were derived from the two Nepalese varieties. Because the two Nepalese varieties lost photoperiod sensitivity and showed accelerated HT, FLT and MAT under short-day conditions (Table 4), it could be assumed that the two Nepalese varieties carry the photoperiod-insensitive allele of *Ppd-B1*. The photoperiod-insensitive alleles of *Ppd-B1* may be the main cause of earliness of HT, FLT and MAT in the two Nepalese varieties. The two photoperiod-insensitive homoeoalleles of *Ppd-1* could independently contribute to segregation of early-flowering individuals in the two F2 populations.

No significant differences in the three flowering-related traits were observed among the F2 individuals with either or both of the photoperiod-insensitive homoeoalleles (Fig. 4), although the LOD scores, contribution to variation and the additive effects of *Ppd-D1* were apparently larger than those of *Ppd-B1* in the SGK/KU-4770 and SGK/KU-180 populations (Table 3). These results did not necessarily correspond with previous reports (Snape et al. 2001, Worland et al. 1998), in which it was demonstrated that plants carrying the *Ppd-D1a* allele are able to undergo earlier flowering than those with the insensitive allele of *Ppd-B1*, *Ppd-B1a*, from CS. The *Ppd-B1a/Ppd-D1a* genotype previously exhibited earlier HT than *Ppd-B1b/Ppd-D1a*, suggesting a significant expression of the *Ppd-B1a* effect in the *Ppd-D1a* genetic background (Seki et al. 2011). On the other hand, it was recently reported that the effect of the photoperiod-insensitive *Ppd-B1a* allele (named *Ppd-B1a.1*) of the cultivar ‘Winter-Abukumawase’ on HT is stronger than that of the CS *Ppd-B1a* allele (*Ppd-B1a.2*) (Nishida et al. 2013). A comparative study of the *Ppd-1* homoeoalleles using doubled haploid lines of common wheat also showed that HT of the genotypes with two or three insensitive homoeoalleles was significantly earlier than that of the single insensitive allele-containing genotypes and that the *Ppd-1* insensitive homoeoalleles exhibit significant interaction (Nishida et al. 2013). The significant interaction between *Ppd-B1* and *Ppd-D1* was also reported using near-isogenic lines for these photoperiod-insensitive alleles (Tanio and Kato 2007). In the present study, significant differences of HT and MAT between the SGK and KU-4770 alleles of the 2B QTL were observed in both of the two mapping populations under the KU-4770 allele of the 2D QTL, whereas no difference of each flowering-related trait was found in either populations under the SGK allele of the 2D QTL (Fig. 4). This observation suggested the presence of interaction between *Ppd-B1* and *Ppd-D1*. It remains unclear whether the function of the photoperiod-insensitive alleles of the Nepalese varieties is identical to that of *Ppd-B1a*. Production of near-isogenic lines of the photoperiod-insensitive alleles may be necessary to clarify the allele identities.

The two Nepalese varieties exhibited earlier HT, FLT and MAT than SGK under short-day conditions, suggesting that early-flowering genes other than *Ppd-B1* exist in the two Nepalese varieties. In the SGK/KU-4770 population, the high LOD score thresholds based on a permutation test might hide minor QTLs for the flowering-related traits. QTL analyses using recombinant inbred lines have been effective in identifying minor QTLs for HD and FLT in common wheat, many of which were associated with narrow-sense earliness (Hanocq et al. 2004, Lin et al. 2008). Novel QTLs for flowering-related traits should be found using recombinant inbred lines of SGK/KU-4770 under controlled growth conditions. In addition, we did not find any QTL for GFP in the present study although wide variations in GFP were observed in the F2 populations (Table 1), which implied that GFP is easily affected by environmental conditions.

In addition to the structural variations in the 5′ and 3′ untranslated regions of *Ppd-B1* (Nishida et al. 2013), copy number variation has been found in *Ppd-B1* and the increased copy number alters *Ppd-B1* gene expression and shortens FLT (Diaz et al. 2012). At the *Ppd-B1* locus of CS, three intact and one truncated copies of *Ppd-B1* exist (Diaz et al. 2012). The *Ppd-B1a* allele-specific PCR primers we used in the present study recognize the truncated copy of *Ppd-B1* (Beales et al. 2007). Our genotyping study, therefore, indicated that KU-4770 includes the *Ppd-B1* truncated copy, whereas no truncated copy was found in KU-180. Real-time PCR analysis to estimate the *Ppd-B1* copy number revealed that KU-4770 and KU-180 respectively contain three and two copies of *Ppd-B1* (Fig. 5), suggesting that the copy number difference between the two Nepalese varieties is due to the presence or absence of the truncated copy and that the two Nepalese varieties include two intact *Ppd-B1* copies. Since no significant difference in HT, FLT and MAT was observed between the two Nepalese varieties under short-day conditions, the truncated copy of *Ppd-B1* apparently has no influence on these flowering-related traits or photoperiod sensitivity. Therefore, the number of intact copies of *Ppd-B1* is important for photoperiod sensitivity in wheat. Some wheat cultivars have been estimated to have two or three copies of *Ppd-B1* (Diaz et al. 2012). We also found copy number variation in *Ppd-B1* of the Nepalese wheat landraces and discovered varieties containing two and three copies of this allele. The intact copy numbers of *Ppd-B1* in the two Nepalese varieties were different from that of CS, meaning that the effect of the photoperiod-insensitive allele in *Ppd-B1* of the Nepalese varieties on flowering-related traits could be inconsistent with that of CS. The increased copy number of *Ppd-B1* pulls up basal accumulation levels of *Ppd-B1* transcripts, putatively resulting in alteration of photoperiod sensitivity (Diaz et al. 2012). To characterize precisely the photoperiod-insensitive alleles of the Nepalese varieties, near-isogenic lines for the insensitive alleles should be produced for future study and *Ppd-B1* gene expression in the near-isogenic lines should be analyzed. To confirm the polymorphisms by the *Ppd-B1* locus reported in Nishida et al. (2013), the nucleotide sequences should be determined in the two Nepalese varieties in a future study.

Wide genetic variation in flowering-related traits have been found in landraces of common wheat (Iwaki et al. 2001, Kato and Yokoyama 1992). Nepalese landraces...
contain both late- and early-heading varieties and their HT is correlated with both photoperiod sensitivity and narrow-sense earliness (Kato and Yokoyama 1992). Our study has suggested that the phenotypes of the two early-flowering varieties of Nepalese wheat are at least partly due to the photoperiod-insensitive allele Ppd-B1, although we failed to identify any significant QTL for narrow-sense earliness. Therefore, wheat landraces are genetic resources for discovery of alleles useful for improvement of HT/FLT of elite cultivars.

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