Research Paper

Genetic analysis of photoperiod sensitivity associated with difference in ecotype in common buckwheat

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Ecotype breeding is a key technology in common buckwheat (*Fagopyrum esculentum* Moench) for the breeding of highly adaptive cultivars and their introduction to other cultivation areas. However, the details of the relationship between photoperiod sensitivity and ecotype remain unclear. Here, we evaluated photoperiod sensitivity in 15 landraces from different parts of Japan, and analyzed quantitative trait loci (QTLs) for photoperiod sensitivity using two F_2 segregating populations derived from the crosses between self-compatible lines ('Kyukei SC2' or 'Buckwheat Norin PL1', early days-to-flowering) and allogamous plants (intermediate or late days-to-flowering). We clarified that (1) photoperiod sensitivity and differences in ecotype are closely related; (2) photoperiod sensitivity is controlled by several QTLs common among population of different ecotypes; and (3) orthologues of *GIGANTEA* and *EARLY FLOWERING 3* will be useful markers in future detailed elucidation of the photoperiod sensitivity mechanism in common buckwheat. This study provides the basis for genomics-assisted breeding for local adaptation and ecotype breeding in common buckwheat.

Key Words: common buckwheat, photoperiod sensitivity, difference in ecotype, genetic analysis, ecotype breeding.

Introduction

Common buckwheat (*Fagopyrum esculentum* Moench, $2n = 16$), a short-day plant and an outcrossing heterostylous pseudocereal crop, is highly adaptable and is grown widely from low to high latitudes in Asia, Europe and North America. Common buckwheat is cultivated throughout most of Japan, and is classified into autumn, intermediate autumn, intermediate summer and summer ecotypes based on the adaptability of the cultivar to the environment in each area. The characteristics of each ecotype and the hypothesis that the ecotype is determined by photoperiod sensitivity have been reviewed by Morishita *et al.* (2020). Because the yield of an ecotype inappropriate for an environment is low (Nagase 2001), choosing the correct ecotypes or improving the suitable ecotype is important for cultivation of common buckwheat. Thus, ecotype breeding is a key breeding technology for the development of highly adaptive cultivars and for their introduction to other cultivation areas.

In long-day cultivation, the phenotypic variation within a common buckwheat population, such as that in flowering time and maturity time, is expanded (Hara and Ohsawa 2013, Minami and Namai 1986). Adaptability to the environment differs between plants with early and late flowering (Minami and Namai 1986). In late-flowering plants, the number of malformed flowers is increased significantly by long day or high temperature (Nagatomo 1961, Nakamura and Nakayama 1950, Sugawara 1958). The ability to set seed and pollen fertility decreases after full flowering time (Nagato *et al.* 1951, Nagatomo 1961, Ohsawa *et al.* 2001, Sugawara and Sugiyama 1954). Although the difference in ecotype in common buckwheat are deeply related to those in photoperiod sensitivity, this relationship has not yet been fully clarified.

Hara *et al.* (2011) demonstrated that photoperiod sensitivity in common buckwheat is controlled by at least three loci, which house candidate genes orthologous to those involved in the photoperiod pathway in *Arabidopsis* (Putterill *et al.* 2004). However, Hara *et al.* (2011) used only a segregating population derived from a cross between two summer-ecotype self-compatible lines; in other common buckwheat ecotypes, loci different from those detected by Hara *et al.* (2011) may control photoperiod sensitivity.

For ecotype breeding in common buckwheat by controlling photoperiod sensitivity, we need to further elucidate the relationship between photoperiod sensitivity and

difference in ecotype by genetic analysis of photoperiod sensitivity. Here, we (1) evaluated photoperiod sensitivity in landraces collected across Japan and in three F_2 segregating populations, and (2) analyzed QTLs for photoperiod sensitivity using two F_2 segregating populations with different photoperiod sensitivities.

Materials and Methods

Evaluation of photoperiod sensitivity and preparation of segregating and mapping populations

To accurately evaluate photoperiod sensitivity, we used 15 landraces (Table 1) grown under various photoperiod conditions throughout Japan and two self-compatible lines, 'Kyukei SC2' (KSC2) and 'Buckwheat Norin PL1' (BNPL1) (Matsui *et al.* 2008); both lines were bred at the National Agricultural Research Center for Kyushu Okinawa Region. Plants were grown and evaluated in an isolated glasshouse (25°C day, 20°C night) at the University of Tsukuba from August 2010. For each of the 15 landraces, a total of 60 seeds (6 seeds × 2 rows × 5 planters) were sown in soil (Peat Pot V, NPK = 200:1000:200 mg/L; Hokkaido Peat Moss Co., Ltd., Hokkaido, Japan) in plastic planters (19 cm × 59 cm × 16 cm; height × length × width); for KSC2 and BNPL1, 36 seeds (6 seeds × 2 rows × 3 planters) were sown. On the basis of previous studies (Hagiwara *et al.* 1998, Hara and Ohsawa 2013, Michiyama and Hayashi 1998, Michiyama *et al.* 2005, Nagatomo 1961, Onda and Takeuchi 1942), the photoperiod was 15 h, which is a long-day condition that causes the expression of differences in photoperiod sensitivity among landraces. Fluorescent lamps for growing plants (Biolux-A FL40SBR-A; NEC Lighting, Ltd., Tokyo, Japan) were used to control the photoperiod. Photoperiod sensitivity evaluation was based on Hara *et al.* (2011) and Hara and Ohsawa (2013) and was performed as follows. The dates of cotyledon development and of the first flower opening in each cluster on the main stem of each plant were recorded. The number of days from the expansion of cotyledons to the first flower opening was defined as days-to-flowering (DTF) and was used as a measure of the photoperiod sensitivity of each plant. Measurements were taken every day until 90 days after sowing, when flowering had ended in all plants. To evaluate differences in photoperiod sensitivity among the landraces, we compared the average DTF by analysis of variance (ANOVA) and Tukey-Kramer tests in JMP v. 6.0 software (SAS Institute Inc., Cary, NC, USA). The relationships of latitude with average DTF and coefficient of variation (C.V.) were examined with Kendall's rank correlation coefficient (τ).

To develop segregating populations, we selected three seed parents derived from three landraces with different DTF—'SOTOYAMA ZAIRAI' (SOT, early DTF), 'KUZUU ZAIRAI' (KUZ, intermediate DTF) and 'MIYAZAKI ZAIRAI' (MIZ, late DTF)—on the basis of the photoperiod sensitivity. BNPL1 and KSC2 (both with early DTF) were selected as pollen parents and were used for artificial pollination. The 'Early-DTF × Early-DTF' (SOT × BNPL1) and 'Intermediate-DTF × Early-DTF' (KUZ × BNPL1) F_2 segregating populations were prepared by crossing SOT (38 DTF) and KUZ (64 DTF) plants, with long-styled flowers, with BNPL1 (27 DTF) (Table 2). The 'Late-DTF × Early-DTF' (MIZ × KSC2) F_2 segregating population was prepared by crossing a MIZ plant with long-styled flower (83 DTF) with KSC2 (25 DTF) (Table 2). The MIZ × KSC2 F_2 segregating population was also used for mapping because

Table 1. Plant materials

Landrace	Abbreviation	Location perfecture	Ecotype[a]	JP No.[a]
ASAHI ZAIRAI	ASA	Gifu	Autumn	41819
MIYAZAKI ZAIRAI	MIZ	Miyazaki	Autumn	48617
KANOYA ZAIRAI	KAN	Kagoshima	Intermediate summer	41846
ZAIRAISHU (GONOHE)	GON	Aomori	Intermediate summer	42885
SOTOYAMA ZAIRAI	SOT	Iwate	Summer	36192
IZUMO (SHIMANE)	IZU	Shimane	Summer	48595
BOTANSOBA[b]	BOT	Hokkaido	Summer	53898
OONO ZAIRAI	OON	Ibaraki	Unkown	36197
KUZUU ZAIRAI	KUZ	Tochigi	Unkown	36207
KAIDA ZAIRAI	KAI	Nagano	Unkown	41815
TANNO HIUSHINAI	TAN	Hokkaido	Unkown	72500
MIYAKO ZAIRAI	MIK	Fukushima	Unkown	Tsukuba Univ.[c]
ASAHIMURA ZAIRAI	ASM	Niigata	Unkown	Tsukuba Univ.[c]
YABAKEI ZAIRAI	YAB	Oita	Unkown	Tsukuba Univ.[c]
IYA ZAIRAI	IYA	Tokushima	Unkown	Tsukuba Univ.[c]

[a] From Genebank Project, NARO.
[b] Cultivar bred at the Hokkaido Agricultural Research Center, NARO.
[c] Population maintained at the University of Tsukuba.

Table 2. F$_2$ segregating and mapping populations

F$_2$ segregating population	Abbreviation	Seed parent		Pollen parent	
		Original landrace	DTF	Original line	DTF
Early-DTF × Early-DTF	SOT × BNPL1	SOT	38	BNPL1	27
Intermediate-DTF × Early-DTF	KUZ × BNPL1	KUZ	64	BNPL1	27
Late-DTF × Early-DTF[a]	MIZ × KSC2	MIZ	83	KSC2	25

[a] This population was used as the mapping population.

it had the largest difference in DTF between parents. The F$_1$ seeds were sown in the isolated glass-house described above, and F$_2$ segregating populations were produced by selfing.

Search for candidate gene regions for photoperiod sensitivity

Genes related to the photoperiod pathways have been identified in many plant species (Hayama and Coupland 2004, Kojima *et al.* 2002, Liu *et al.* 2001a, Nemoto *et al.* 2003, Yano *et al.* 2000). Hara *et al.* (2011) suggested the existence of a similar photoperiod pathway in common buckwheat. To test this suggestion and to determine the genetic relations between these orthologous genes and QTLs, we selected 16 genes that are related to flowering time in *Arabidopsis* (Putterill *et al.* 2004) (**Supplemental Table 1**). The sequences were obtained from the National Center for Biotechnology Information database and used as queries in BLASTP searches in the Buckwheat Genome Database (BGDB; Yasui *et al.* 2016). Matches were considered to be significant when the smallest sum probability (*P*) was <0.0001 and the bit scores was >100. The retrieved orthologous regions were considered candidate photoperiod sensitivity gene regions, and single nucleotide polymorphism (SNP)-based markers for the candidate genes were generated as in Hara *et al.* (2011).

Marker development and linkage map construction

We developed new expressed sequence tag (EST)-based markers using the 863 cDNA clones (139 inflorescence-derived clones, designated the Fest_F group, and 724 leaf-derived clones, designated the Fest_L group) which were generated by Hara *et al.* (2011). For developing new EST-based markers (cleaved amplified polymorphic sequence markers, CAPS; derived cleaved amplified polymorphic sequence markers, dCAPS; and insertion/deletion [Indel] markers, which produced large differences in the length of polymerase chain reaction [PCR] products), primer design and searches for polymorphism (SNPs, insertions and deletions) between mapping population parents were done as in Hara *et al.* (2011). To compare the linkage map developed in this study and a buckwheat high-density linkage map developed Yabe *et al.* (2014), which includes many DNA micro-array markers, neighbor markers were developed near the micro-array markers located at both ends of each linkage group (LG) in the high-density linkage map. Neighbor

markers (CAPS, dCAPS and Indel markers) were also developed for large intervals between markers on the linkage map after integration. Candidate gene markers (including 3 described in Hara *et al.* 2011), EST-based markers (including 76 described in Hara *et al.* 2011), neighbor markers and 180 microsatellite markers (Konishi and Ohnishi 2006) were assayed in the MIZ × KSC2 F$_2$ mapping population (*n* = 384). A framework linkage map for QTL analysis was constructed as in Hara *et al.* (2011) in JoinMap v. 4.0 software (Van Ooijen 2006). Total DNA of both parental lines and of each of the F$_2$ segregating plants was extracted as follows: ~200 mg milled leaf tissue was mixed with 750 μl lysis buffer (0.3% sodium dodecyl sulfate, 20 mM Tris-HCl [pH 8.0 at 25°C], 5 mM EDTA, 400 mM NaCl), incubated at 65°C for 10 min and centrifuged at $17,800 \times g$ for 2 min. The supernatant was diluted 1:500 with ultrapure water (Milli-Q) and was used for PCR. PCR amplification, sequencing, restriction enzyme treatment, electrophoresis and gel imaging were carried out as described in Hara *et al.* (2011). Integration with the high-density linkage map (Yabe *et al.* 2014) was performed by using the neighbor markers as anchor markers.

QTL analysis for photoperiod sensitivity in segregating populations

Plants were grown in an isolated glasshouse at Tsukuba University as described above. Photoperiod sensitivity under short-day (12 h) conditions was surveyed from January 2015, and that under long-day conditions from August 2014. The photoperiod sensitivity of each plant of the three F$_2$ segregating populations was measured as described above. DNA markers were used to screen for polymorphisms among parents. The genotype of each plant of the F$_2$ segregating population was determined using polymorphic markers. PCR amplification, restriction enzyme treatment, electrophoresis and gel imaging were carried out as described in Hara *et al.* (2011). Interval mapping, cofactor analysis and multiple-QTL method (MQM) analysis were performed using the photoperiod sensitivity of each plant of the KUZ × BNPL1 and MIZ × KSC2 F$_2$ segregating populations (the SOT × BNPL1 was not analyzed), the genotype at each marker and the created linkage map as described in Hara *et al.* (2011) in JoinMap software and MapQTL v. 5.0 software (Van Ooijen 2004).

Results

Photoperiod sensitivity of landraces collected across Japan

Under long-day conditions, the average DTF varied among landraces and showed a continuous distribution from 26.2 days (SOT) to 60.2 days (MIZ) (**Table 3**). A negative correlation was found between latitude and average DTF ($\tau = -0.4519$, $P < 0.01$). The distribution of DTF within each landrace was also continuous; among individual plants, the earliest flowering (16 DTF) was found in 'BOTANSOBA' (BOT) and the latest flowering (86 DTF) in 'ASAHIMURA ZAIRAI'(ASM) (**Supplemental Fig. 1**). Since the average DTF of landraces was distributed continuously and the seed set ability was not measured in this study, it was difficult to determine the ecotype of each landrace. No significant correlation was found between latitude and C.V. ($\tau = -0.1635$, $P > 0.05$); the within-landrace diversity of photoperiod sensitivity was highest in the middle-latitude regions of Japan, followed by the low- and high-latitude regions (**Table 3**).

Search for candidate photoperiod sensitivity gene regions in the buckwheat genome database

We detected a total of 38 scaffolds with high sequence identity to the selected 16 Arabidopsis photoperiod pathway genes (**Supplemental Table 1**). Multiple orthologous regions other than the *TIMING OF CAB EXPRESSION 1* (*TOC1*), *EARLY FLOWERING 3* (*ELF3*) and *GIGANTEA* (*GI*) genes were identified, clearly indicating that buckwheat contains a set of orthologous genes associated with the photoperiod pathway from light perception to floral

Table 3. Days-to-flowering of each population under long-day conditions

Landrace	Latitude of prefectural office location	Average DTF (mean ± SD)	C.V.[a] (%)	Tukey-Kramer test
SOT	39° 42′	26.2 ± 3.96	15.1	a
TAN	43° 03′	28.8 ± 4.46	15.5	a
GON	40° 49′	29.2 ± 5.40	18.5	a
BOT	43° 03′	30.7 ± 4.72	15.4	ab
IZU	35° 28′	36.6 ± 8.16	22.3	bc
OON	36° 20′	38.0 ± 10.18	26.8	c
KUZ	36° 33′	38.4 ± 13.09	34.1	c
IYA	34° 03′	41.3 ± 10.94	26.5	cd
MIK	37° 45′	42.4 ± 11.53	27.2	cde
KAN	31° 33′	44.9 ± 10.46	23.3	de
KAI	36° 39′	45.6 ± 12.04	26.4	de
ASA	35° 23′	47.7 ± 10.92	22.9	de
YAB	33° 14′	48.7 ± 12.32	25.3	e
ASM	37° 54′	55.9 ± 17.16	30.7	f
MIZ	31° 54′	60.2 ± 14.15	23.5	f

[a] Coefficient of variation = standard deviation/average value × 100.

induction. A total of 15 candidate gene markers for photoperiod sensitivity genes were developed and tested for linkage map construction (**Supplemental Tables 1**, **2**).

Marker development and linkage map construction

We developed 116 novel EST-based markers (13 from the Fest_F group, 103 from the Fest_L group) from among the original 863 EST regions, and 73 neighbor markers (Ne_FE group) based on DNA micro-array markers developed by Yabe *et al.* (2014). Among these, we confirmed polymorphisms between parents of the mapping population (MIZ × KSC2, $n = 384$) at 17 candidate genes (including 2 described in Hara *et al.* 2011), 192 EST-based markers (including 76 described in Hara *et al.* 2011), the 73 above neighbor markers and 180 simple sequence repeat (SSR) markers (Konishi and Ohnishi 2006). Out of the total 462 markers, polymorphisms were confirmed at 275 markers (15 candidate genes, 141 EST-based, 73 neighbor and 46 SSR markers). Of these, 229 were SNP-based markers (including 155 CAPS and 57 dCAPS markers) and 17 were Indel markers. Because genotype classification was difficult for 26 markers, they were used as dominant markers; the other markers were co-dominant. Information on the 229 markers with polymorphisms (excluding 46 SSR markers) is shown in **Supplemental Table 2**.

Through the linkage mapping of these markers, the positions of 269 of them (15 candidate genes, 141 EST-based, 67 neighbor and 46 SSR markers) were estimated; 6 other markers showed a departure from Mendelian segregation ratios (**Fig. 1**, **Supplemental Table 2**). The linkage map consisted of 8 LGs ranging from 64.9 cM (LG8) to 113.5 cM (LG1), and covered 752.5 cM in total (**Fig. 1**). The maximum interval between adjacent markers was 14.3 cM in LG6 (Fes3265 at 82.0 cM to Fes4151 at 96.3 cM), with an average of 2.8 cM. This linkage map was integrated with a high-density linkage map (Yabe *et al.* 2014) using neighbor markers: P1_1 and P2_1 were designated as LG1, P1_2 and P2_2 as LG2, P1_3 and P2_3 as LG3, P1_4 and P2_4 as LG4, P1_5 and P2_5 as LG5, P1_6 and P2_6 as LG6, P1_7 and P2_7 as LG7, and P1_8.1, P1_8.2 and P2_8 as LG8.

QTL analysis for photoperiod sensitivity

In all F_2 segregating populations, cultivation under long-day conditions resulted in a significant ($P < 0.01$) delay in average DTF, and median DTF was also delayed (**Fig. 2**). The segregation of photoperiod sensitivity in all three F_2 populations under long-day conditions showed a wide distribution, and C.V. was higher than under short-day conditions (**Fig. 2**). SOT × BNPL1 and KUZ × BNPL1 F_2 segregating populations showed superdominance in early flowering; SOT × BNPL1 and especially MIZ × KSC2 showed superdominance in late flowering (**Fig. 2**). We performed QTL analysis using two F_2 populations, KUZ × BNPL1 and MIZ × KSC2.

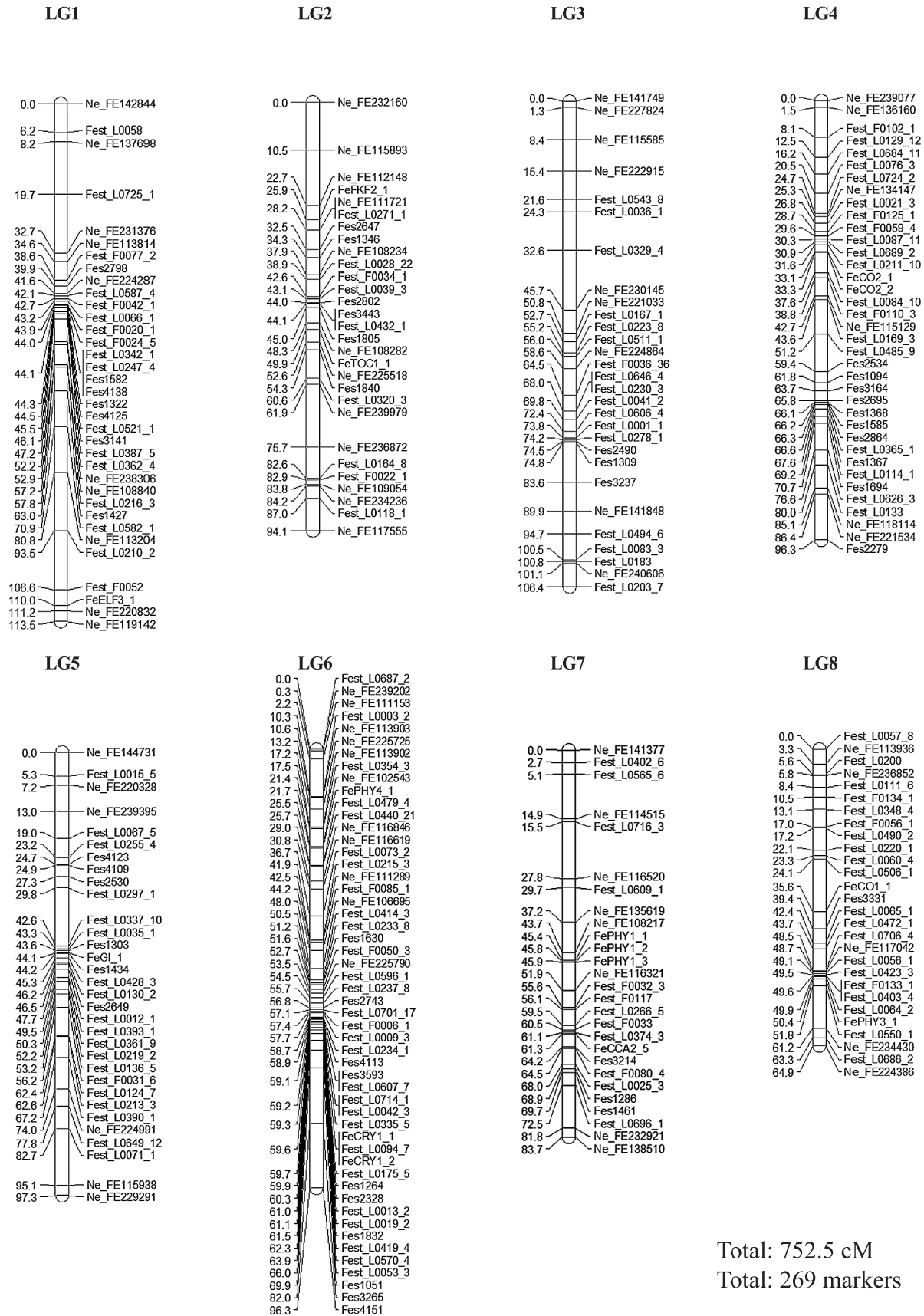Of the 282 markers (excluding 180 SSR markers), 79

Fig. 1. Linkage map. LG, linkage group. 'Fest_F' and 'Fest_L' are EST-based markers. 'Ne_FE' markers are based on DNA micro-array markers developed by Yabe *et al.* (2014). 'Fes' markers are based on SSR-based markers developed by Konishi and Ohnishi (2006).
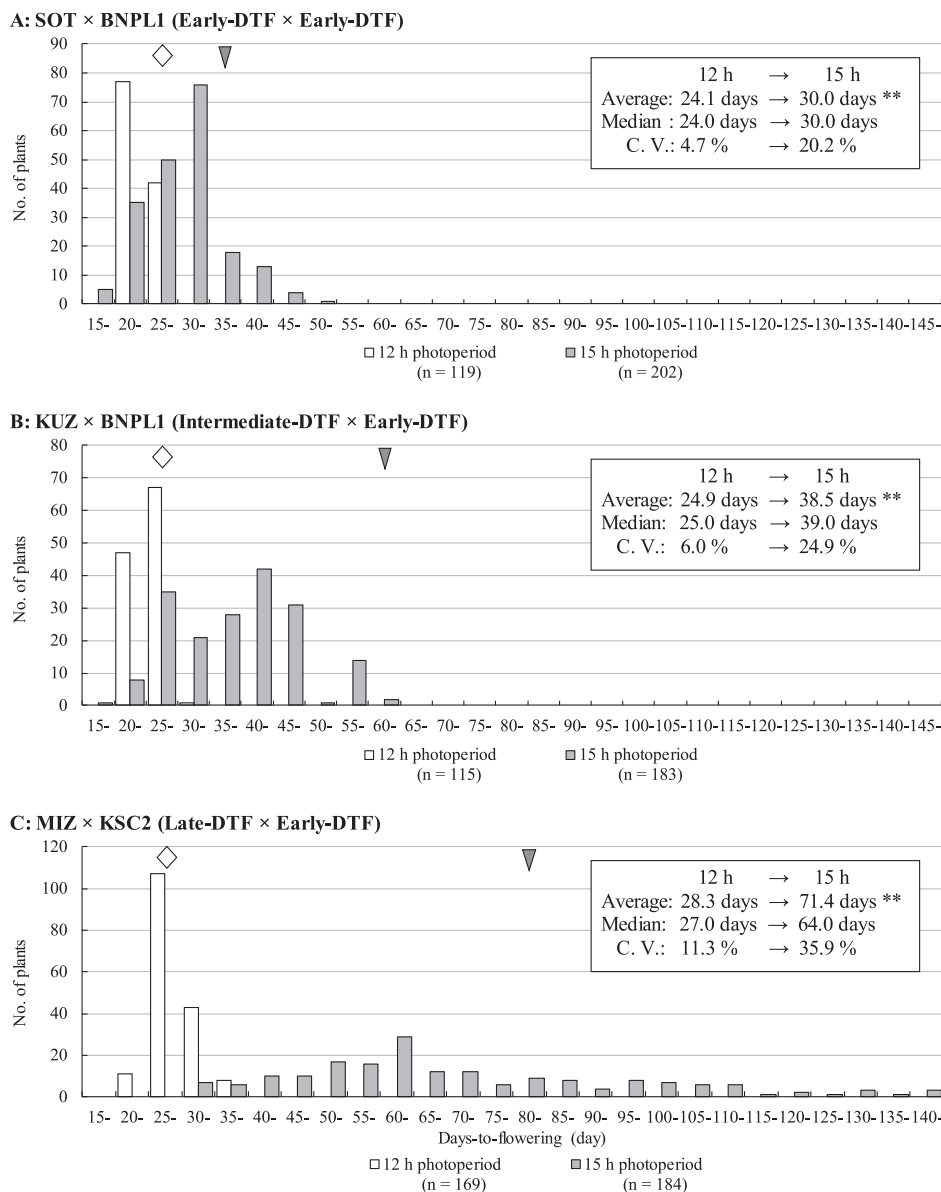
Total: 752.5 cM
Total: 269 markers

Fig. 2. Distribution of days-to-flowering (DTF) in F_2 segregating populations. (A) SOT × BNPL1 (Early-DTF, 38 DTF × Early-DTF, 27 DTF), (B) KUZ × BNPL1 (Intermediate-DTF, 64 DTF × Early-DTF, 27 DTF), and (C) MIZ × KSC2 (Late-DTF, 83 DTF × Early-DTF, 25 DTF). White bars, short day (12 h photoperiod); gray bars, long day (15 h photoperiod). White rhombi and gray arrowheads indicate the DTF of parent plants (**Table 2**). **Significant at the 1% level compared with short-day conditions.

were polymorphic among the parents of the KUZ × BNPL1 F_2 segregating population. We performed QTL analysis with the genotypes of these markers and with DTF at 12 and 15 h as trait values. In interval mapping, two regions in LG1 (108.6–111.2 cM) and LG4 (13.5 cM) at 12 h and one region in LG1 (62.8–113.5 cM) at 15 h were associated with photoperiod sensitivity (at LOD score thresholds of >3.5 at 12 h and >3.3 at 15 h; $P < 0.05$). In cofactor analysis, a significant difference ($P < 0.02$) was detected at two markers (FeELF3_1 in LG1 and Fest_L0021_3 in LG4) at 12 h and at three markers (FeELF3_1 in LG1, FeTOC1_7 in LG2 and Fest_L0337_10 in LG5) at 15 h. In MQM analysis, we detected *QTL for flowering time under 12 h photo-*

period in Intermediate-DTF × Early-DTF population 1 (*qFT12hI×E_1*) and *qFT12hI×E_2* (for *qFT12hI×E_1*, nearest DNA marker was Fest_L0724_2 in LG4; for *qFT12hI×E_2*, the nearest maker was FeELF3_1 in LG1), which showed high LOD scores at 12 h (>3.6, $P < 0.05$; **Table 4**, **Fig. 3A**), and *QTL for flowering time under 15 h photoperiod in Intermediate-DTF × Early-DTF population 1* (*qFT15hI×E_1*) to *qFT15hI×E_3* (*qFT15hI×E_1*, nearest maker FeELF3_1 in LG1; *qFT15hI×E_2*, Fest_L0337_10 in LG5; *qFT15hI×E_3*, Ne_FE225518 in LG2), which showed high LOD scores at 15 h (>3.3, $P < 0.05$; **Table 4**, **Fig. 3B**). The candidate genes for photoperiod sensitivity—*Fagopyrum esculentum EARLY FLOWERING 3* (*FeELF3*;

Table 4. Quantitative trait loci (QTLs) for photoperiod sensitivity detected in each F_2 segregating population by multiple-QTL analysis

Photo-period	F_2 segregating population	LG[a]	Position (cM)	Detected QTL	Nearest DNA marker	Neighborhood candidate gene (distance to QTL)	LOD score	Additive[b] effect	Dominant[b] effect	PVE[c] (%)
12 h	KUZ × BNPL1 (Intermediate-DTF × Early-DTF)	4	24.7	qFT12hI×E_1	Fest_L0724_2	None identified	7.5	−4.2	−4.4	28.6
		1	110.3	qFT12hI×E_2	FeELF3_1	FeELF3 (0.0 cM)	3.8	0.6	−0.9	15.6
	MIZ × KSC2 (Late-DTF × Early-DTF)	6	54.5	qFT12hL×E_1	Fest_L0596_1	FeCRY1 (5.1 cM)	32.8	−2.6	−3.1	56.6
		8	61.2	qFT12hL×E_2	Ne_FE234430	None identified	4.3	−0.9	−0.8	4.7
		3	100.5	qFT12hL×E_3	Fest_L0083_3	None identified	3.7	−0.8	−0.6	3.8
15 h	KUZ × BNPL1 (Intermediate-DTF × Early-DTF)	1	110	qFT15hI×E_1	FeELF3_1	FeELF3 (0.0 cM)	23.2	7.5	6.9	44.1
		5	42.6	qFT15hI×E_2	Fest_L0337_10	FeGI (1.5 cM)	4.9	−8.9	−9.1	19.2
		2	52.6	qFT15hI×E_3	Ne_FE225518	FeTOC1 (2.7 cM)	4.4	−3.2	0.5	15.4
	MIZ × KSC2 (Late-DTF × Early-DTF)	5	43.6	qFT15hL×E_1	Fes1303	FeGI (0.5 cM)	15.0	−17.6	0.8	24.5
		3	68.0	qFT15hL×E_2	Fest_L0230_3	None identified	5.3	−8.4	−6.9	7.6
		8	49.9	qFT15hL×E_3	Fest_L0064_2	FePHY3 (0.5 cM)	4.8	−10.7	−5.5	6.9

[a] Linkage group.
[b] Values for the 'KSC2' or 'BNPL1' genotype.
[c] Percentage of total phenotypic variance explained by the QTL.

interval, 0.0 cM), *Fagopyrum esculentum GIGANTEA* (*FeGI*; 1.5 cM) and *Fagopyrum esculentum TIMING OF CAB EXPRESSION 1* (*FeTOC1*; 2.7 cM)—were present near the QTLs *qFT12hI×E_2/qFT15hI×E_1*, *qFT15hI× E_2* and *qFT15hI×E_3*, respectively (**Table 4**, **Fig. 1**). The BNPL1 alleles of all QTLs except *qFT12hI×E_2* and *qFT15hI×E_1* had a negative additive effect on photoperiod sensitivity (**Table 4**). Both QTLs detected at 12 h had a complete dominant effect on early flowering (*qFT12hI×E_1* −4.4 days, *qFT12hI×E_2* −0.9 days; **Table 4**). Two QTLs detected at 15 h had a complete dominant effect on late flowering (*qFT15hI×E_1* 6.9 days) and early flowering (*qFT15hI×E_2* −9.1 days; **Table 4**).

Of the 462 markers, 275 were polymorphic among the parents of the MIZ × KSC2 F_2 segregating population. We performed QTL analysis with the genotypes of these markers, and with DTF at 12 and 15 h as trait values. In interval mapping, one region in LG6 (10.6–96.3 cM) at 12 h and three regions in LG3 (66.5–81.8 cM), LG5 (20.0–87.1 cM) and LG8 (44.0–45.0 cM) at 15 h were associated with photoperiod sensitivity (LOD score >3.6; $P < 0.05$). In cofactor analysis, a significant difference ($P < 0.02$) was detected at three markers (Fest_L0183 in LG3, Fest_L0596_1 in LG6 and Ne_FE234430 in LG8) at 12 h, and at three other markers (Fest_L0230_3 in LG3, Fes1303 in LG5 and Fest_L0064_2 in LG8) at 15 h. In MQM analysis, we detected *QTL for flowering time under 12 h photoperiod in Late-DTF × Early-DTF population 1* (*qFT12hL×E_1*) to *qFT12hL×E_3* (*qFT12hL×E_1*, nearest marker Fest_L0596_1 in LG6; *qFT12hL×E_2*, Ne_FE234430 in LG8; *qFT12hL×E_3*, Fest_L0083_3 in LG3), which showed high LOD scores at 12 h (>3.5, $P < 0.05$; **Table 4**, **Fig. 3A**), and *QTL for flowering time under 15 h photoperiod in Late-DTF × Early-DTF population 1* (*qFT15hL×E_1*) to *qFT15hL×E_3* (*qFT15hL×E_1*,

Fes1303 in LG5; *qFT15hL×E_2*, Fest_L0230_3 in LG3; *qFT15hL×E_3*, Fest_L0064_2 in LG8), which showed high LOD scores at 15 h (>3.7, $P < 0.05$; **Table 4**, **Fig. 3B**). Three candidate genes for photoperiod sensitivity— *Fagopyrum esculentum CRYPTOCHROME 1* (*FeCRY1*; interval, 5.1 cM), *FeGI* (0.5 cM) and *Fagopyrum esculentum PHYTOCHROME 3* (*FePHY3*; 0.5 cM)—were present near the QTLs *qFT12hL×E_1*, *qFT15hL×E_1* and *qFT15hL ×E_3*, respectively (**Table 4**, **Fig. 1**). The KSC2 alleles of all QTLs had a negative additive effect on photoperiod sensitivity (**Table 4**). All QTLs detected at 12 h had a complete dominant effect on early flowering (*qFT12hL×E_1* −3.1 days, *qFT12hL×E_2* −0.8 days, *qFT12hL×E_3* −0.6 days; **Table 4**). Two QTLs detected at 15 h had an incomplete dominant effect on early flowering (*qFT15hL ×E_2* −6.9 days, *qFT15hL×E_2* −5.5 days; **Table 4**).

Discussion

Relationship between photoperiod sensitivity and difference in ecotype in common buckwheat

Using landraces collected across Japan, we found a negative correlation ($\tau = -0.4519$, $P < 0.01$) between latitude and photoperiod sensitivity (**Table 3**). This result is similar to that of Ujihara and Matano (1974). However, it was difficult to strictly classify the landraces into the four ecotypes because the distribution of DTF in each landrace and among the landraces was continuous (**Supplemental Fig. 1**). Moreover, no correlation ($\tau = -0.1635$, $P < 0.05$) was found between latitude and the within-landrace diversity of photoperiod sensitivity (C.V.) (**Table 3**), and although the low-latitude landraces were more diverse than the high-latitude landraces, the middle-latitude landraces had the highest diversity (**Table 3**). In Japan, the summer ecotype may have differentiated from the autumn ecotype through
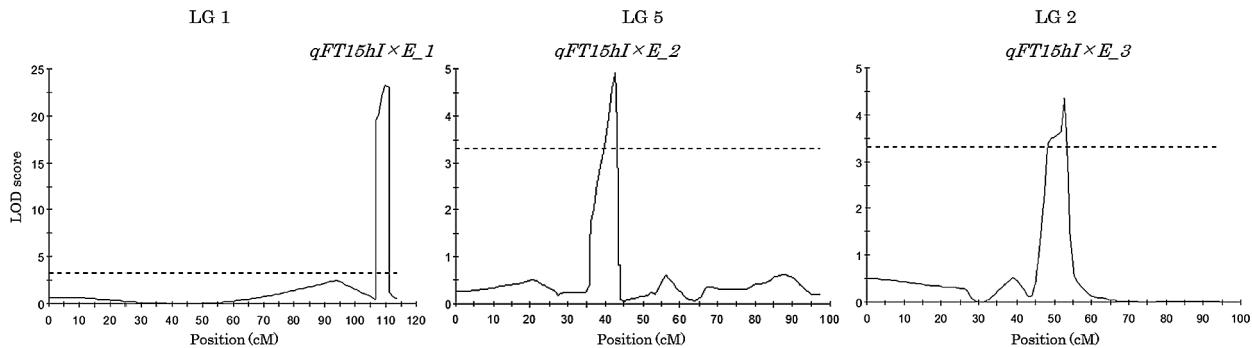
A

KUZ × BNPL1 (Intermediate-DTF × Early-DTF)
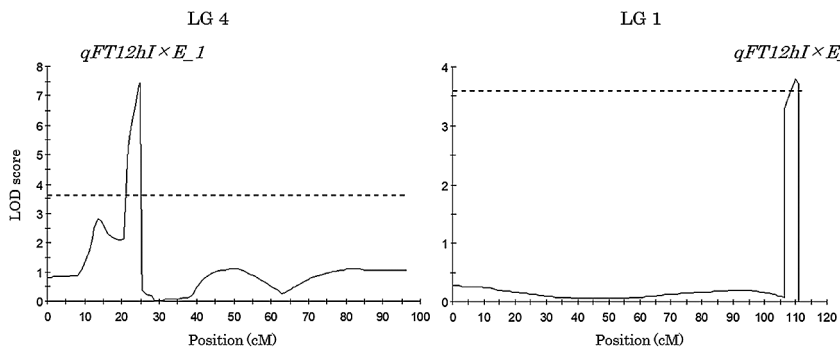
MIZ × KSC2 (Late-DTF × Early-DTF)

B
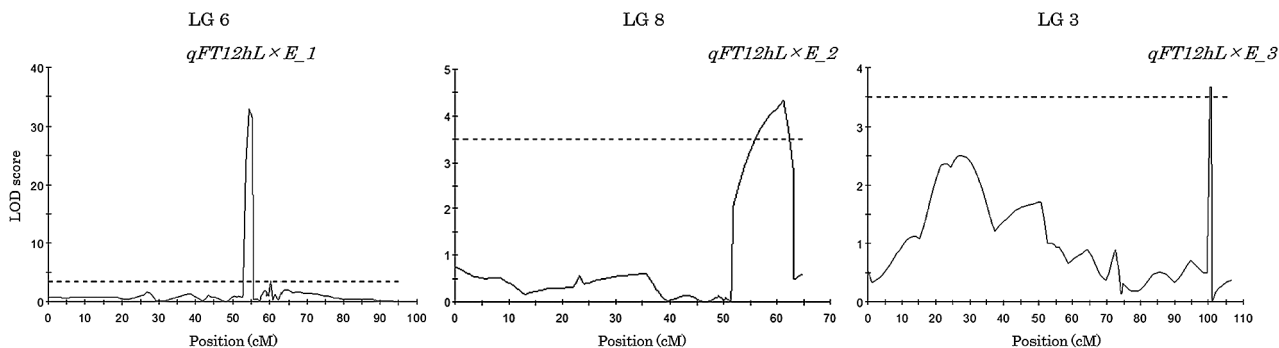
KUZ × BNPL1 (Intermediate-DTF × Early-DTF)

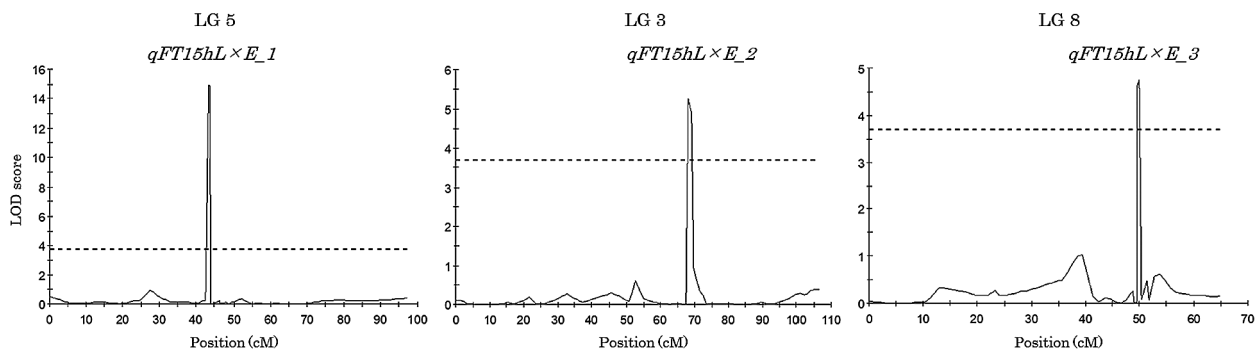

MIZ × KSC2 (Late-DTF × Early-DTF)

Fig. 3. QTLs detected in multiple-QTL analysis. (A) QTLs detected under short-day (12 h photoperiod) conditions. (B) QTLs detected under long-day (15 h photoperiod) conditions. The QTL likelihood map for each linkage group (LG) was obtained by using the MQM procedure of MapQTL. Linkage group number is indicated at the top of each graph. Horizontal dashed lines indicate the significant ($P < 0.05$) LOD score threshold.

an intermediate ecotype (Hara and Ohsawa 2013, Matano and Ujihara 1979, Minami and Namai 1986, Morishita *et al.* 2020, Ujihara and Matano 1978). In line with this hypothesis, the genetic and phenotypic diversities of summer-ecotype (high-latitude) landraces tend to be lower than those of autumn-ecotype (low-latitude) landraces, and the genetic structure differs between summer and autumn ecotypes (Hara and Ohsawa 2013, Iwata *et al.* 2005, Michiyama and Hayashi 1998, Onda and Takeuchi 1942). However, the present study, which accurately evaluated the photoperiod sensitivity of landraces collected across Japan, showed that the middle-latitude landraces are the most diverse. To clarify what kinds of ecological changes have occurred in Japan, it will be necessary to conduct further research using landraces from all over the world.

Genetic analysis of photoperiod sensitivity associated with differences in common buckwheat ecotypes

We constructed a linkage map that included 15 candidate gene markers likely related to photoperiod sensitivity in 8 LGs containing 269 markers and covering 752.5 cM in total; the average interval between adjacent markers was 2.8 cM (**Fig. 1**). Some linkage maps with 8 LGs have been previously developed for common buckwheat. Yasui *et al.* (2004) constructed a linkage map that coverd a total of 508.3 cM and contained 223 amplified fragment-length polymorphism markers. Yabe *et al.* (2014) constructed a high-density linkage map that coverd a total of 800.4 cM and contained 1631 contigs and 4657 DNA micro-array markers. The linkage map constructed here contained markers located at both ends of each LG in the high-density linkage map (Yabe *et al.* 2014) as neighbor markers, and the markers covered almost the entire common buckwheat genome. Therefore, it will be suitable for comprehensive and efficient genome-wide searching for QTLs for photoperiod sensitivity.

In the MQM analysis with the 12 h photoperiod, we detected two QTLs in the KUZ × BNPL1 F_2 segregating population and three in the MIZ × KSC2 F_2 segregating population (**Table 4**, **Fig. 3**). With the 15 h photoperiod, we detected three QTLs in each F_2 segregating population (**Table 4**, **Fig. 3**). Hara *et al.* (2011) and Minami (1985) suggested that photoperiod sensitivity of common buckwheat is controlled by multiple genes, and our study supports this suggestion. The distribution of DTF in the MIZ × KSC2 F_2 segregating population at 15 h was broad (30 to 140 days), and the values of the percentage of total phenotypic variance explained by the detected QTLs were lower than those in the KUZ × BNPL1 F_2 segregating population (**Fig. 2**, **Table 4**). These results suggest the existence of unidentified minor QTLs, and DTF in the MIZ × KSC2 F_2 segregating population at 15 h may be controlled by the accumulation of the effects of such QTLs.

Our QTL analysis under long-day conditions identified *qFT15hI×E_2* (KUZ × BNPL1 population) and *qFT15hL×E_1* (MIZ × KSC2 population) as located extremely close

to each other (**Table 4**, **Fig. 3B**). Hara *et al.* (2011) detected three QTLs for photoperiod sensitivity (*FeELF3*, Fest_L0606_4 and Fest_L0337_6) in an Early-DTF × Early-DTF segregating population. Fest_L0337_6 was derived from the same cDNA clone as Fest_L0337_10 used here (DNA marker near *qFT15hI×E_2* and *qFT15hL×E_1*; **Table 4**). Therefore, we presume that *qFT15hI×E_2* and *qFT15hL×E_1* are important QTLs for the difference in ecotype in common buckwheat.

Although not confirmed in the MIZ × KSC2 population, *qFT12hI×E_2* and *qFT15hI×E_1* identified in the KUZ × BNPL1 population are the same QTL common to both long- and short-day conditions, and explained a large percentage of total phenotypic variance under long-day conditions (**Table 4**, **Fig. 3**). A QTL near FeELF3_1 (DNA marker nearest to *qFT12hI×E_2* and *qFT15hI×E_1*) has also been identified by Hara *et al.* (2011); in both studies, it had an additive effect in the direction of late flowering, unlike other QTLs (in MIZ × KSC2, the LOD value was not significant at 2.4, but the additive effect was in the direction of early flowering). These facts suggest that the QTL corresponding to *qFT12hI×E_2* and *qFT15hI×E_1* is related to the difference in photoperiod sensitivity among ecotypes.

Candidate genes of the photoperiod pathway (*FeELF3*, *FeCRY1*, *FeGI*, *FeTOC1* and *FePHY3*) were present near 7 of the 11 QTLs identified here (**Table 4**). In *Arabidopsis*, *ELF3* and *TOC1* connect the circadian clock to the photoperiod pathway (Covington *et al.* 2001, Liu *et al.* 2001b, Putterill *et al.* 2004, Wang *et al.* 1997, Wang and Tobin 1998), and *CRY* (*CRYPTOCHROME*) and *PHY* (*PHYTO-CHROME*) connect light perception to it (Putterill *et al.* 2004). *GI* connects the long-day pathway to it (Fowler *et al.* 1999, Park *et al.* 1999, Putterill *et al.* 2004). Our results suggest that genes orthologous to photoperiod pathway genes regulate photoperiod sensitivity in common buckwheat, as proposed by Hara *et al.* (2011). These candidate genes may be useful for the future detailed elucidation of the photoperiod sensitivity mechanism in common buckwheat. In particular, we consider *FeGI* and *FeELF3*, located in the immediate vicinity of four QTLs (*qFT15hI×E_2*, *qFT15hL×E_1*, *qFT12hI×E_2* and *qFT15hI×E_1*; **Table 4**), as remarkable candidate genes important for the differences among ecotypes.

In summary, we have demonstrated the relationship between photoperiod sensitivity and difference in ecotype using landraces collected across Japan and cultivated under long-day conditions, and identified some QTLs associated with photoperiod sensitivity. Although unresolved points remain, our results confirm the previous hypotheses that differences among ecotypes are due to differences in photoperiod sensitivity. From the results of this study, we expect that genomics-assisted ecotype breeding for local adaptation in common buckwheat will be made possible by development of DNA markers related to photoperiod sensitivity.

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