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QTL analysis of photoperiod sensitivity in common buckwheat by using markers for expressed sequence tags and photoperiod-sensitivity candidate genes

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Photoperiod sensitivity is an important trait related to crop adaptation and ecological breeding in common buckwheat (*Fagopyrum esculentum* Moench). Although photoperiod sensitivity in this species is thought to be controlled by quantitative trait loci (QTLs), no genes or regions related to photoperiod sensitivity had been identified until now. Here, we identified QTLs controlling photoperiod sensitivity by QTL analysis in a segregating F₄ population ($n=100$) derived from a cross of two autogamous lines, 02AL113(Kyukei SC2)LH.self and C0408-0 RP. The F₄ progenies were genotyped with three markers for photoperiod-sensitivity candidate genes, which were identified based on homology to photoperiod-sensitivity genes in *Arabidopsis* and 76 expressed sequence tag markers. Among the three photoperiod-sensitivity candidate genes (*FeCCA1*, *FeELF3* and *FeCOL3*) identified in common buckwheat, *FeELF3* was associated with photoperiod sensitivity. Two EST regions, FesL_L0606_4 and FesL_L0337_6, were associated with photoperiod sensitivity and explained 20.0% and 14.2% of the phenotypic variation, respectively. For both EST regions, the allele from 02AL113(Kyukei SC2)LH.self led to early flowering. An epistatic interaction was also confirmed between FesL_L0606_4 and FesL_L0337_6. These results demonstrate that photoperiod sensitivity in common buckwheat is controlled by a pathway consisting of photoperiod-sensitivity candidate genes as well as multiple gene action.

Key Words: common buckwheat, EST markers, photoperiod-sensitivity candidate genes, photoperiod sensitivity, QTL analysis.

Introduction

Common buckwheat (*Fagopyrum esculentum* Moench, $2n=16$), a pseudocereal, has been widely cultivated in Asia, Europe and North America. In Japan, common buckwheat has been cultivated throughout most of the country, with cultivars classified into three ecotypes: summer, intermediate, and late-summer. The late-summer cultivars are cultivated in low-latitude regions where photoperiod is around 12 to 14 h during the cropping season, and their flowering is generally sensitive to photoperiod. The summer cultivars are cultivated in high-latitude regions where photoperiod is around 14.5 to 15.5 h during the cropping season (summer crop season), and they are generally insensitive to photoperiod. Intermediate cultivars show moderate photoperiod sensitivity (Namai 1990). Common buckwheat is considered to be a short-day plant, with the threshold between flowering and non-flowering at a photoperiod of about 14.5 h

(Nagatomo 1961). Under long-day conditions, (i.e., photoperiod longer than 14.5 h), late-summer cultivars showed wide variation in flowering time, which resulted in low yield because of the increased incidence of malformed flowers and the decrease in ripening rate in the late-flowering individuals (Nagatomo 1961, Nakamura and Nakayama 1950, Sugawara 1958). The intermediate and summer cultivars are considered to have been derived from the late-summer cultivars through both natural and artificial selection (Matano and Ujihara 1979). Minami and Namai (1986a, 1986b) suggested that summer cultivars differentiated from late-summer cultivars through the selection of early-flowering plants under long-day conditions; this selection was part of the domestication of buckwheat for climatic adaptation to the northern part of Japan.

Iwata *et al.* (2005) reported that allelic richness (i.e., the degree of polymorphism of genetic markers) has decreased in Japanese common buckwheat varieties cultivated in high-latitude regions during selection for early flowering time, and they suggested that the decrease of allelic richness at neutral loci was caused by bottleneck effects caused by directional selection based on photoperiod sensitivity. Ohsawa

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(1997) reported that the heritability of flowering period was high (95.1%), suggesting that photoperiod sensitivity was a key feature of adaptation and differentiation in common buckwheat. Photoperiod-sensitivity genes, however, have not been identified in common buckwheat.

Minami (1985) suggested that photoperiod sensitivity in common buckwheat was controlled by a number of genes. In several crops, photoperiod sensitivity is considered to be inherited quantitatively (Putterill *et al.* 2004, Yano *et al.* 2001). Genetic regulation of flowering induced by photoperiod has been studied in *Arabidopsis* and rice (*Oryza sativa* L.), and many genes related to photoperiod sensitivity have been detected as quantitative trait loci (QTLs) and isolated by positional cloning (Corbesier and Coupland 2005, Hayama and Coupland 2003). These genes compose the photoperiod-sensitivity pathway (Putterill *et al.* 2004), and have been confirmed among many species as orthologous genes (Hayama *et al.* 2002, 2003, Hayama and Coupland 2003, Kojima *et al.* 2002, Liu *et al.* 2001a, Nemoto *et al.* 2003, Yano *et al.* 2000). Moreover, photoperiod sensitivity in both long-day and short-day plants appears to be controlled by a common mechanism (Putterill *et al.* 2004). Based on this information, we hypothesized that photoperiod sensitivity of common buckwheat would also be controlled by the action of multiple genes orthologous to those of the photoperiod-sensitivity pathways in other species.

To avoid the complex genetic patterns caused by the allogamous (cross-pollinating) reproductive system of most common buckwheat, we used autogamous (self-pollinating) lines showing differences in photoperiod sensitivity for simplicity of analysis. We used expressed sequence tag (EST) markers derived from cDNAs of candidate photoperiod-sensitivity genes to detect photosensitivity QTLs most efficiently.

In the present study, QTLs related to photoperiod sensitivity in common buckwheat were identified in the following three steps: (1) evaluation of variation in photoperiod sensitivity between two autogamous parental lines, (2) linkage map development using EST markers, and (3) QTL analysis for photoperiod sensitivity.

Materials and Methods

Photoperiod sensitivity of parental lines

For detecting QTLs related to photoperiod sensitivity, we employed two parental lines, 02AL113(Kyukei SC2)LH.self (KYU) and C0408-0RP (CAN). KYU is an autogamous line bred at the National Agricultural Research Center for Kyushu Okinawa Region, and CAN is an autogamous line bred at Kade Research Ltd. (Canada). In general, differences in photoperiod sensitivity between these two lines was visible under photoperiods longer than 14.5 h. We used two photoperiod conditions, a natural long-day condition (day length of ~14.5 h) and an artificial long-day condition (15.5 h), to detect the difference in photoperiod sensitivity between KYU and CAN. For the natural long-day condition, 60 seeds each of KYU and CAN were sowed by 12 seeds per

one planter (19 × 59 × 16 cm) and cultivated in growth cabinet without supplemental light at the University of Tsukuba. For the artificial long-day condition, 12 seeds each of KYU and CAN were sowed in a planter and cultivated in a growth cabinet with day length of 15.5 h. Metal halide lamps (MLBOC400C-U) (Mitsubishi Electric Corp., Tokyo, Japan) were used to control the day length at artificial long-day condition.

The investigation of photoperiod sensitivity was performed as follows. The dates of cotyledon development and of the first flower flowering were recorded for each individual. The number of days from the expansion of cotyledons to the first flower flowering was defined as days-to-flowering, which was used as a measure of the photoperiod sensitivity of each individual. The measurements were performed every day until 100 days after sowing. At 100 days after sowing, individuals that had not yet flowered were classified as 'non-flowering' and these were excluded from the following analyses. To evaluate differences in photoperiod sensitivity between the populations, we performed analysis of variance (ANOVA) and *t* tests based on the days-to-flowering under each photoperiod condition by using the program JMP 6.0 (SAS Institute Inc., NC, USA).

cDNA library construction and search for photoperiod-sensitivity candidate gene regions

We developed sets of 362 cDNA clones derived from inflorescences and 1920 cDNA clones derived from leaves. 362 cDNA clones derived from inflorescences was provided by the Niigata University of Pharmacy and Applied Life Sciences. To develop 1920 cDNA clones derived from leaves, we used 'Miyazakizairai' (late-summer cultivar) for plant material and sampled leaves six times at 4-h intervals from individuals that were either early flowering or non-flowering under the 15.5 h day-length condition because it is clarified that amount of photoperiod-sensitivity genes expression in *Arabidopsis* was changed over time. Total RNA was extracted by using RNeasy Plant Mini Kit (Qiagen Inc., CA, USA) following manufacturer's instructions. cDNA library was directionally constructed (5' SfiIA, 3' SfiIB) in pDNR-LIB using the Creator SMART cDNA Library Construction Kit (Clontech Laboratories, Inc., CA, USA) and size-fractionated by using CHROMA SPIN+TE-1000 (Clontech Laboratories, Inc.). Libraries were manually arrayed in 96-well microtiter plates. Glycerol stocks of overnight cultures were prepared in 96-well format. Plasmid DNAs were extracted and BigDye Terminator (Life Technologies Corp., NY, USA) cycle sequenced on ABI PRISM 3730xl Genetic Analyzer (Life Technologies Corp.) by using conventional procedures and the following primers: M13 forward primer (5'-GTAAAACGACGGCCAGT-3') and M13 reverse primer (5'-AAACAGCTATGACCATGTTCA). Vector sequences and low quality regions were trimmed from EST sequences manually before removing overlapping regions by using BLASTN, and then 863 clones (139 inflorescence-derived clones, designated as the Fest_F group, and 724 leaf-derived

clones, designated as the Fest_L group) were used for further analysis. To search for photoperiod-sensitivity candidate gene regions, a homology search against previously reported sequences to *Arabidopsis* was performed by using tBLASTX. Matches were considered to be significant when the smallest sum probability (p) was less than 0.0001 and bit scores were greater than 100.

Development of the mapping population

We developed a mapping population consisting of 100 F₄ lines derived by single-seed descent from a cross between KYU as the female parent and CAN as the male parent. Because we observed stunted growth in the F₅ generation, we used the F₄ generation as the population for linkage mapping and QTL analysis.

Total DNA samples of both parental lines and the F₄ lines were extracted by the following method. About 100 mg leaf tissue was mixed with 500 μ l lysis buffer (0.3% sodium dodecyl sulfate, 20 mM Tris-HCl (pH 8.0 at 25°C), 5 mM EDTA, 400 mM NaCl), and incubated at 65°C for 10 min with 5 μ l of 10 mg/ml Proteinase K (Wako Pure Chemical Ind., Ltd., Osaka, Japan) and 100 mg/ml RNase A (Qiagen Inc.). The DNA was purified with chloroform-octanol (24:1) and precipitated with isopropanol. The pellet was washed with 70% ethanol and resuspended in 100 μ l Tris-EDTA buffer.

Marker development and linkage map construction

Primer sets corresponding to three candidate genes (identified in the homology search described above) and randomly selected 170 ESTs (78 ESTs from the Fest_F cDNA clones, 92 ESTs from the Fest_L cDNA clones) among 863 cDNA clones were designed by using PRIMER3 software (Supplemental Table 1). PCR was performed with candidate gene and EST primers and genomic DNA isolated from KYU and CAN as the template. The amplified products were then sequenced to check whether the target region had been amplified. PCR was performed under the following conditions: 30 ng of genomic DNA was amplified by PCR in a 30 μ l final reaction volume containing 6 μ M each of the forward and reverse primers, 6 mM each of deoxynucleotide triphosphate (dNTPs), 10 \times PCR buffer, and 0.75 units of Blend Taq DNA polymerase (TOYOBO Co., Ltd., Tokyo, Japan). PCR was performed by using the Mastercycler gradient PCR system (Eppendorf Scientific Inc., Hamburg, Germany). The PCR program consisted of a preliminary denaturation step of 2 min at 94°C; followed by 40 cycles of 30 s at 94°C (denaturation), 30 s at gradient temperatures (50 to 70°C) (annealing), and 2 min at 72°C (extension); with a final extension for 7 min at 72°C. PCR products were analyzed by electrophoresis in 8% acrylamide (29:1 acrylamide:bis-acrylamide) gels. For sequencing, the PCR products within a single band were purified by using the QIAquick PCR purification kit (Qiagen Inc.) and then sequenced by using the BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Life Technologies Corp.) and an ABI PRISM

3100 Genetic Analyzer (Life Technologies Corp.).

Primer sets that produced large differences in amplicon length (detected on 8% acrylamide gels) between parental lines were developed as EST markers. In cases where no difference in amplicon length could be detected, we searched for single-nucleotide polymorphisms (SNPs) by sequencing, and developed cleaved amplified polymorphic sequence (CAPS) markers in regions where SNPs were found within restriction-enzyme recognition sites. When no SNP could be found within the recognition sequences of restriction enzymes, derived cleaved amplified polymorphic sequence (dCAPS) markers were developed instead. The SNP searches were performed by using Phred/Phrap/Consed software (Gordon and Green 1998). The selection of restriction enzymes to detect SNPs and design of mismatch primers for dCAPS was conducted with dCAPS Finder 2.0 (Neff *et al.* 2002; <http://helix.wustl.edu/dcaps/dcaps.html>).

To detect polymorphisms of CAPS and dCAPS markers, PCR products produced by using EST primers or dCAPS primers were digested with a corresponding restriction enzyme in 12 μ l reaction volumes containing 10 μ l of the PCR products, 6 units restriction enzyme, and 1.2 μ l of the supplied 10 \times buffer. The reaction temperature was set to the recommended temperature for each restriction enzyme, and the reaction time was 3 h. PCR and electrophoresis conditions were the same as described above.

The candidate gene and EST markers were assayed in the F₄ mapping population. A framework linkage map for QTL analysis was constructed with the software JOINMAP 4.0 (Van Ooijen 2006) by using genotype data for each marker. The fit to the Mendelian segregation ratio for each marker was tested with the Chi-square test ($P < 0.05$). Markers were assigned to linkage groups with a logarithm of odds (LOD) threshold of 3.0.

QTL analysis

The F₄ population was cultivated in a growth cabinet and scored for photoperiod sensitivity under a 15.5 h photoperiod, as described above. QTL analysis was performed by using the software MAPQTL version 5 (Van Ooijen 2005). Interval mapping analysis was performed (Lander and Botstein 1989) to locate preliminary QTL positions on the map. Interval mapping was used to select markers significantly associated with the trait to constitute an initial set of cofactors. A backward-elimination procedure was applied to the initial set of cofactors. Only significant markers ($P < 0.02$) were used as cofactors in the multiple QTL method (MQM) (Jansen 1993, Jansen and Stam 1994) analysis for QTL detection. After the selection of cofactors, MQM analysis was performed. A 1000-permutation test was applied to each data set to establish the LOD value to be used for determining the significance ($P < 0.05$) of identified QTLs (Churchill and Doerge 1994). The phenotypic variance explained by a single QTL was calculated as the square of the partial correlation coefficient (r^2) with the observed variable, adjusted for cofactors. When candidate gene markers

could not be mapped, the association between each of candidate gene markers and photoperiod sensitivity was analyzed with ANOVA. To evaluate digenic interactions between pairs of QTLs, we performed two-way ANOVA for two-locus QTL genotypes, as represented by the genotypes of markers nearest QTLs. These ANOVA were calculated by using the program JMP 6.0.

Results

Natural variation in photoperiod sensitivity between autogamous lines

The frequency distributions of days-to-flowering of KYU and CAN are shown in Fig. 1. Under the natural long-day condition, the days-to-flowering distributions of the KYU (range 22–38 days) and CAN (21–32 days) populations were similar (Fig. 1A), and the means (24.7 and 24.9 for KYU and CAN, respectively) were not significantly different ($P = 0.681$) (Fig. 1A). On the other hand, under the artificial long-day condition with 15.5 h day length, the mean days-to-flowering in KYU (mean 24.6; range 24–31 days) was significantly shorter ($P < 0.0001$) than that of CAN (mean 48.0; range 41–59 days) (Fig. 1B).

Search for photoperiod-sensitivity candidate gene regions

In tBLASTX searches against the reference sequences of proteins in *Arabidopsis*, three Fest_L EST regions (Fest_L0268, Fest_L0327 and Fest_L0352) showed high homology with photoperiod-sensitivity genes in *Arabidopsis*. Fest_L0268 showed high homology with *CONSTANS-LIKE 3* (*COL3*), *CONSTANS-LIKE 1* (*COL1*) and *CONSTANS-LIKE 2* (*COL2*); Fest_L0327, with *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*); and Fest_L0352, with *EARLY FLOWERING 3* (*ELF3*) (Table 1). Among the sequences with homology to multiple genes, Fest_L0268 showed the highest homology with *COL3*, and Fest_L0327 showed the highest homology with *CCA1*. We considered these three EST regions as candidate genes related to photoperiod sensitivity in common buckwheat, and designated them *FeCOL3*, *FeCCA1*, and *FeELF3*, respectively. None of the Fest_F regions showed high homology to photoperiod genes in *Arabidopsis*.

Development of linkage map using EST markers

We developed primers for the three candidate genes

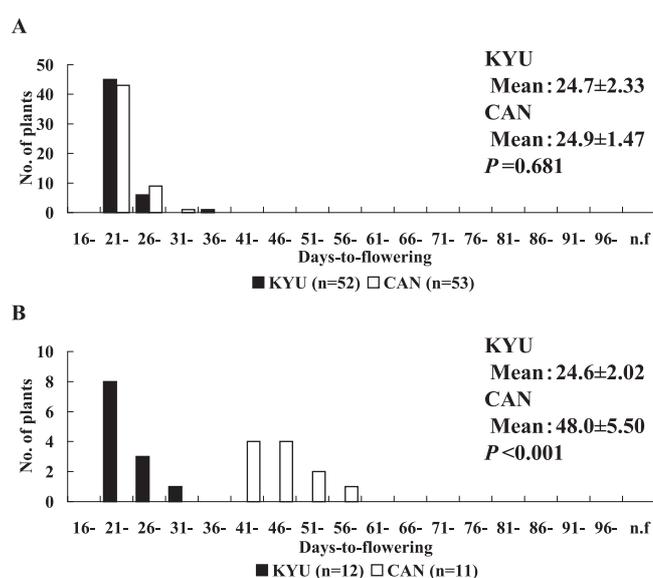


Fig. 1. Variation in days-to-flowering between the parental lines. (A) Variation in days-to-flowering of parental lines under natural long-day condition (~14.5 h day length). (B) Variation in days-to-flowering of parental lines under artificial long-day condition (15.5 h day length). KYU, 02AL113(Kyukei SC2)LH.self., CAN, C0408-0 RP; P value in each figure showed significance of difference by t-test.

(*FeCOL3*, *FeCCA1* and *FeELF3*) and randomly selected 170 ESTs (78 from the Fest_F group, 92 from the Fest_L group) from among the original 863 EST regions (Supplemental Tables 1 and 2 showed results of BLASTX for randomly selected 170 EST regions). Among these, we confirmed polymorphism between KYU and CAN for the three candidate gene and 115 ESTs regions. Sixteen of the 115 polymorphic EST regions included large indels, whereas the three candidate gene and 99 ESTs regions included small indels and SNPs. The 16 EST regions containing large indels were used as length markers; in addition, we developed three candidate gene and 60 SNP-based markers (39 CAPS and 21 dCAPS markers) from among the 99 ESTs markers. Information on the three candidate gene markers and 76 EST markers is shown in Table 2.

Through the linkage mapping of these markers, the positions of 63 EST markers were estimated. Nine other EST markers showed departure from Mendelian segregation ratios, and four EST markers could not be mapped (Table 2 and Fig. 2). The map consisted of 9 linkage groups (LG)

Table 1. Confirmed sequences showing high homology with photoperiod-sensitivity genes in *Arabidopsis*

EST region	Gene product	Gene ID	Bit score	E-value
Fest_L0268	<i>COL3</i> (<i>CONSTANS-LIKE 3</i>); protein binding / transcription factor/ zinc ion binding	817016 COL3	124	4.00E-29
	<i>COL1</i> (<i>constans-like 1</i>); transcription factor/ zinc ion binding	831442 COL1	112	2.00E-25
	<i>COL2</i> (<i>constans-like 2</i>); transcription factor/ zinc ion binding	821298 COL2	112	2.00E-25
Fest_L0327	<i>CCA1</i> (<i>CIRCADIAN CLOCK ASSOCIATED 1</i>); DNA binding / transcription activator/ transcription factor/ transcription repressor	819296 CCA1	142	4.00E-34
	<i>LHY</i> (<i>LATE ELONGATED HYPOCOTYL</i>); DNA binding / transcription factor	839341 LHY	137	5.00E-33
Fest_L0352	<i>ELF3</i> (<i>EARLY FLOWERING 3</i>); protein C-terminus binding / transcription factor	817134 ELF3	104	1.00E-22

Table 2. Summary information for the three candidate genes and 76 EST markers

Maker name	Type of marker	SNP region	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product length (bp)	Annealing temp. (°C)	Restriction enzyme
<i>FeCOL3</i>	CAPS	intron	TCAAGACTCAGCTGGTGAACG	GGACGGATCAGAAAACTCGTC	713	62	BclI
<i>FeCCAI</i>	CAPS	exon	TCGAGCCAAAGCAGTTACTACG	CCACAAGAGGAACGGTCAAC	901	62	Hpy188I
<i>FeELF3^b</i>	CAPS	intron	TTGGGAGTTCGGGGATGAG	TGCCTTGCTCTTTCCCTGGT	1280	62	DraI
Fest_F0005 ^b	EST	-	CAAGCCAAACAAGCTGGAGAA	AATGGGAGAAATGCTTAGTTGCTTAC	810	62	-
Fest_F0035	EST	-	CAGTAAGGCAGACGGTTGA	TCATTTGCCAGATCTCACTGGA	148	58	-
Fest_F0049	EST	-	GTGAAAATATGTTTTCTTTTAGAGG	TTCTTGCTATGAGCACTGAAA	123	54	-
Fest_F0052 ^a	EST	-	GGACGGACATGGCTGATTAC	TGACCAACCACACAAAAACAC	163	58	-
Fest_F0056 ^a	EST	-	AAGGATCACAGA AACCGGAA	CAAGCCAAATGATCCATGACAAAAC	266	62	-
Fest_F0057	EST	-	TCTTTGTTGTCCAAGATTGCTG	CCAAATCGTAATCATAAGCGTTCC	314	54	-
Fest_F0078	EST	-	AAAACCCCTCTTTCTCCCC	TGGCCTTGATTCACCTTCT	217	62	-
Fest_F0090	EST	-	CAGCAGTTCATGCATCTGTAA	CGATGATGATGATGGTGGAGA	348	58	-
Fest_F0095	EST	-	ATTACTGCCACCTCATCAGACC	AGATCGCTCAGTATCGTGCC	699	62	-
Fest_F0110	EST	-	GAGGAGGAACAAGACGCACA	TCAAGCCAGTGACATACCACAA	1354	62	-
Fest_F0117	EST	-	GAGTCTTTTCCAAAGCCCATC	TGGAGAGGTCAAATCCAGCC	702	58	-
Fest_F0119 ^b	EST	-	GGGAGGATTCATTTCTACAGCA	CCAATGTCAGGAGTTAAGAGAG	183	58	-
Fest_F0129	EST	-	TGGACGAGCTTTCATAGAG	TCCTTGAACATAAATCACAATG	161	54	-
Fest_F0138 ^b	EST	-	CACATACCAACCAAACTCAAATACA	GCCCAGATATCGTTTGTCTC	184	58	-
Fest_L0080	EST	-	CTTCAGTTTTCCAGTTCCTTCCA	CAGCGCAATGTTCCCTTC	787	54	-
Fest_L0433	EST	-	GAAAGAGTTCAAACAAGAGC	AACACCCTCTTCAAAGTTC	1128	58	-
Fest_F0031_1	CAPS	exon	CAAAGCACGTCCAAACAACA	GGATAATGGCGGTGTCAAAA	788	58	Hpy188I
Fest_F0053_2	CAPS	exon	GCGTTATTTCCCGACTGATG	GATGCACATAGACATGGCTGG	212	62	BsuRI
Fest_F0076_9 ^a	CAPS	exon	TTCTGTTAGTTAAAATGAAAATACA	ATAGCAAACGGACACAAAATAA	613	54	HindIII
Fest_F0077_2	CAPS	exon	TTGGGAGAAATCAAAATACG	TCGAAAGGATTTGTTGCAGTTG	498	62	ApaLI
Fest_F0081_7	CAPS	exon	CATTACAACACACCGCATCGG	AGCAAGGCAAGCTCTTCTGG	369	58	BshNI
Fest_F0083_8	CAPS	intron	CATGGCAAACTCCCTTTC	CGTACCACGATCAATTCAGATCA	2010	54	MspI
Fest_F0087_2	CAPS	exon	CAGACCTCAAATGTTCCACCAG	GAAGAGGCAAAGGAGGCAAG	520	62	TaqI
Fest_F0100_15 ^a	CAPS	intron	TCCAAATCGCGTTGACAGAA	GAAAATGCCGTCCACACAAAG	431	58	AvaII
Fest_F0102_1 ^a	CAPS	exon	GGGCAGACTCGGTGCTATT	GGTGAAGGGATTGTGGCTGT	291	62	HinfI
Fest_F0139_3	CAPS	exon	ATTCTGGTGTCACTGCTCTTA	GAGCCATTGATCGTGATTC	357	62	TaqI
Fest_L0030_1	CAPS	intron	AAAGAAGGAAAAGGCTCCCC	GATGCAACTATCCATGCCCC	1135	58	BsuRI
Fest_L0041_2	CAPS	exon	GTCCACAGGAGGAAGGCAC	CGTGAACACACCAAATACGACC	527	62	TaqI
Fest_L0046_2	CAPS	exon	CAITGTTCTCAAGCAGCAAAA	GGATACAGGGTTGGTGGAGG	373	62	HinfI
Fest_L0064_2	CAPS	exon	CGGAGAATGGCTTCCAAGAG	GAGAAGTGGCATCTGGCTGG	659	66	TaqI
Fest_L0101_1	CAPS	intron	GGCATCCATGTTTAGCTCTGG	ACCTCAAGGGCACGGTTCT	392	62	HinfI
Fest_L0124_7	CAPS	exon	CCACCTGGCTCTCGTCTTC	GAAACGCCCAACCCATACC	376	66	MspI
Fest_L0130_2	CAPS	exon	CTGAACCAACACAAAACGATCAA	TGGTTTGGAAACAAGGAAGTCG	1386	62	HinfI
Fest_L0136_5	CAPS	exon	AAGCAGCACCTTCACAGCAA	CGAAGAAGCTGGGTCGTAG	431	66	BsuRI
Fest_L0169_3	CAPS	exon	AACCTCGCATTTTCAGTCCC	ACAGCAATACTCCGGCTTC	629	60	MspI
Fest_L0186_3	CAPS	exon	CCACACACACAAAAGCACC	CACGAACACTAGGACGACTCTGAA	492	62	TaqI
Fest_L0211_5 ^b	CAPS	exon	GGGAATCGGAGTTGGTTACTCTC	CTGGTGGCTTTGGATGGTTT	525	62	TaqI
Fest_L0219_2	CAPS	exon	GCTTGTAGATGGGGTGTGG	CGTTTCGGCAGAGTTTCATC	717	62	StyI

Table 2. (continued)

Maker name	Type of marker	SNP region	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product length (bp)	Annealing temp. (°C)	Restriction enzyme
Fest_L0266_5	CAPS	exon	TCTCTCATCCCAAAAACCCAA	TAATCCAACGGCTCAAAACCA	391	60	HhaI
Fest_L0292_1	CAPS	exon	ACCCGTCCCAAGTCAAAGAAA	AAAGTCATAAACCCCTGATGCC	479	60	BsoBI
Fest_L0377_4	CAPS	exon	CGTTTTTCATGGTGTGGTTCC	CGGAGAAACGGGTAAGGTGT	576	62	EcoRV
Fest_L0426_12	CAPS	intron	ATCGATTTTCGAGGGGTTTG	TATTTGAGGAGGCCATTTCTG	700	62	MseI
Fest_L0432_1	CAPS	exon	CAAAGCAGGCAGAGAAAGCA	GCAGATGCAAAAATCAACTTCCC	559	62	AvaII
Fest_L0480_1	CAPS	intron	TGTGCAACTCAGAGATTAAAC	TGGATGCCAGTTCAAAGGTC	597	62	BglII
Fest_L0490_2	CAPS	exon	GAGCAGGGTGTGCTCTCT	TAGCGTCTCCAAAATGTCCTG	808	62	HhaI
Fest_L0494_6	CAPS	exon	TCCCAATCTCTCCTCTCCTCC	AGCACCAACAAACGCAAAAC	603	62	TaqI
Fest_L0506_1	CAPS	intron	CGTCGTAITTCGCTCTCC	GCAITTCATCATTTGGGCTGT	870	62	DraI
Fest_L0542_3 ^a	CAPS	exon	GCGAATTCGGTTTTCACAAA	TACCTCGCCGACTACAACA	427	62	KpnI
Fest_L0543_1	CAPS	exon	TTGATTCCTGGGCAACAGAG	ACTTGACAGCAATGCAAAAG	488	60	MspI
Fest_L0556_6	CAPS	exon	CCCTAATAACCCCGAAACCC	AAAGCCTTCTCAGCAGTGTC	481	62	MspI
Fest_L0578_2	CAPS	exon	GGCCCGTCAAAACCAAAA	CCCTGAACCTCAATTTGGCAG	375	62	SacI
Fest_L0606_4	CAPS	exon	TGACAAAGATGAAGGACTGGA	AGCAGGAGAGCCTGTTGATTT	308	62	TaqI
Fest_L0609_1 ^a	CAPS	exon	TGCCTCTAACTGTTCCAAATCC	TCGTGACATCATCTTCCGT	227	62	AseI
Fest_L0706_2	CAPS	exon	GGGGAGTGAGGAAAACCAACA	ACAGAAAACCAATCAATCCACC	514	62	BstXI
Fest_L0712_10	CAPS	exon	TAACAACCCGCTCTCTTCCC	ATCCATGACACGCTCTCGCTC	592	62	HinfI
Fest_F0032_2	dCAPS	intron	ACGCAITTCAAAATTCAAATGCA	GAAGAAGCTGGCGAAGAAGA	154	52	EcoT221
Fest_F0034_1	dCAPS	intron	TACTTGAATCCAAATCCTTAATAATTAGGATC	CGGGATTCCTTTGGTGGTTT	151	58	BamHI
Fest_F0043_1	dCAPS	exon	TACCGGCTTCCCAATGTGCAA	TTCCGGAAAGAGGAAATCACCC	180	62	HindIII
Fest_F0050_3	dCAPS	exon	GTGTCCTCGAAAGTCCACC	GCCCTTAGGGAGATCAGGAA	182	60	SylI
Fest_F0075_2	dCAPS	exon	AGAAAGTGAAGATGACTTTAA	ATTTCCCATTTGGCTTTT	158	52	DraI
Fest_F0079_1	dCAPS	exon	TCGATTTTACCTTCACCCGCCATGGTCAA	CAGTTTAAACGGCAAAACATACCCCTTTT	138	56	HindIII
Fest_F0080_4	dCAPS	exon	TGAGGAGGTGAGGCTTACC	TGTTTTGTGGGTTTTTTTGACTG	158	58	HhaI
Fest_F0085_1 ^a	dCAPS	exon	GACCAAAGAAACAAATTTTA	TTGGTTGGTCAATTAATCTAT	151	52	DraI
Fest_F0101_1	dCAPS	exon	AGGATAAAAGAAAGAAAGAGTAAAGATT	CCTGGACATGCATAAATAAA	176	58	HinfI
Fest_F0108_1	dCAPS	exon	GAAAGTGTGTAGACAGAT	TAGTTTGGTAAATACTAGC	224	50	HinfI
Fest_F0112_3	dCAPS	exon	TACTAATACAAACTTAATTT	CTTATGATAGTGTCTAAT	198	42	DraI
Fest_F0127_1	dCAPS	exon	CGCCGTGCGCCGCTCAGGAGTCTCCGTC	TCCAATCGCTCAGAAGGATCCGAAGG	139	62	HindIII
Fest_F0133_1	dCAPS	exon	AAAGTCTCTTGTGATCTTCAAG	AGCTCAACAACCTTTCACTT	175	50	HindIII
Fest_F0134_1	dCAPS	exon	CAAGAACCTCCAAAGTAAAGTTCTAATATGC	GCTTACAAATGCAACTTTACAACCGA	168	58	EcoT221
Fest_L0053_3	dCAPS	exon	AATACAGTACTGGTGGGTC	CTCATCACCTTCTTCTTC	164	54	MspI
Fest_L0129_12 ^a	dCAPS	exon	CGCAGCTTGCTATTTGCTCC	CCCAAAAGATCCCAATCCCTC	177	56	MspI
Fest_L0242_1	dCAPS	exon	CGCTATGCCGCCCTTACTGCG	GCCATGTCGGGAAAGAAAGGTC	167	54	HhaI
Fest_L0329_3	dCAPS	intron	CATCCATCTTGATTTCAAG	TACACTCAGGAGTCAAGAAC	171	56	ApoI
Fest_L0337_6	dCAPS	exon	ATCTTCAGGCCTAATGTTAA	AATCAGCAAACCTCTTAAGC	289	54	HindIII
Fest_L0472_1	dCAPS	exon	GGATGAGGTGGGCCAGGTGCCAGAGCTGT	TTCAAGGGAGAAAATGGAGGCCATGA	123	56	BstXI
Fest_L0582_7	dCAPS	exon	GAAAACATGTTCTTAGATAT	AGAAAGGATAGCTTAAATG	154	50	EcoRV

^a Marker showed segregation ratio that was significantly different from the expected Mendelian ratio.

^b Marker could not be mapped (Fig. 2).

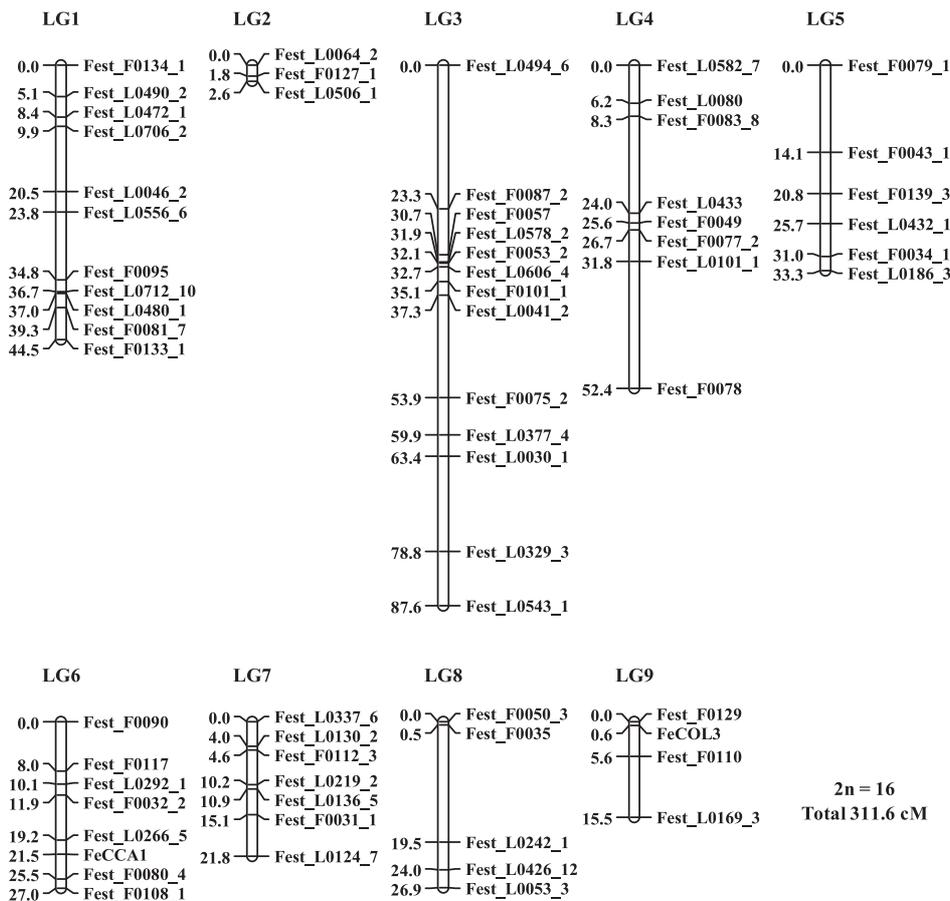


Fig. 2. Linkage map of common buckwheat.

ranging from 2.6 cM (LG2) to 87.6 cM (LG3) and covered 311.6 cM in total. The segregation ratios for the three candidate gene markers were not significantly different from the expected Mendelian ratios. *FeCOL3* and *FeCCA1* markers were mapped to LG9 and LG6, respectively, but *FeELF3* could not be mapped (Table 2 and Fig. 2).

QTL analysis for photoperiod sensitivity

The segregation of photoperiod sensitivity in the F_4 progenies was observed as a wide but unimodal distribution. Transgressive segregation in the direction of late flowering was observed (Fig. 3).

When we performed interval mapping, two regions were associated with photoperiod sensitivity: one in LG3 (25.3 to 50.3 cM) and one in LG7 (0 to 15.1 cM) (LOD score >2.4, $P < 0.05$). The results of cofactor analysis indicated a significant difference ($P < 0.02$) at eight markers (Fest_L0490_2 and Fest_L0472_1 in LG1, Fest_L0606_4 in LG3, Fest_F0077_2 and Fest_L0101_1 in LG4, Fest_L0337_6 in LG7 and Fest_L0242_1 and Fest_L0053_3 in LG8). In the MQM analysis, two QTLs (Fest_L0606_4 and Fest_L0337_6 in LG3 and 7, respectively) showed relatively high LOD values (LOD score >2.4, $P < 0.05$) (Table 3 and Fig. 4). For each of these two QTLs, the KYU alleles had negative additive effects on days-to-flowering (−8.2 days

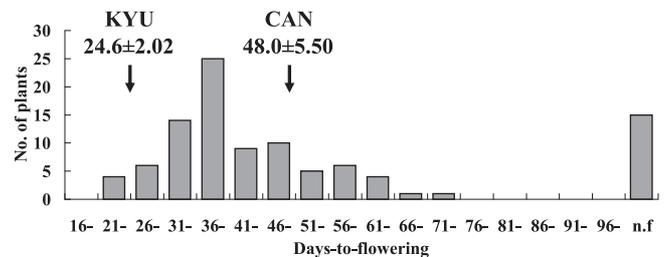


Fig. 3. Distribution of days-to-flowering in F_4 progeny (n = 100) of a cross of 02AL113(Kyukei SC2)LH.self (KYU) × C0408-0 RP (CAN). Arrows indicate means ± SD for each parent. n.f., non-flowering.

and −6.8 days, for Fest_L0606_4 and Fest_L0337_6, respectively) and explained about 20.0% and 14.2% of the phenotypic variation in the F_4 progenies, respectively.

In the candidate gene markers, *FeCCA1* and *FeCOL3* showed low LOD values (LOD score 0.13 and 0.11, respectively) as a result of the MQM analysis (Table 3). *FeELF3* could not be mapped. Thus, we searched for associations between *FeELF3* and photoperiod sensitivity by using the ANOVA. Table 4 shows the result of the ANOVA for association between the *FeELF3* and photoperiod sensitivity. It was shown that *FeELF3* was significantly related to photoperiod sensitivity ($P = 0.028$). For *FeELF3* markers, CAN

Table 3. Quantitative trait loci (QTLs) detected for photoperiod sensitivity in the 02AL113(Kyukei SC2)LH.self (KYU) × C0408-0 RP (CAN) F₄ population by multiple QTL analysis

LG	Position (cM)	Locus	LOD	Additive effect	PVE (%)
3	34.0	Fest_L0606_4	5.2	-8.2	20.0
7	0.0	Fest_L0337_6	3.8	-6.8	14.2
6	21.5	<i>FeCCA1</i>	0.13	1.2	0.4
9	0.6	<i>FeCOL3</i>	0.11	-1.1	0.4
U	-	<i>FeELF3</i>	-	-	-

U, unknown genome location

Table 4. Quantitative trait loci (QTLs) detected for photoperiod sensitivity in the 02AL113(Kyukei SC2)LH.self (KYU) × C0408-0 RP (CAN) F₄ population by ANOVA. KYU and CAN indicate average of days-to-flowering for the 02AL113(Kyukei SC2)LH.self allele and homozygosity for the C0408-0 RP allele, respectively.

LG	Position (cM)	Locus	<i>F</i> value	<i>P</i> value	Average of days-to-flowering	
					KYU	CAN
U	-	<i>FeELF3</i>	3.72	0.028	51.5	42.9
3	34.0	Fest_L0606_4	10.40	<0.001	39.3	54.6
7	0.0	Fest_L0337_6	6.17	0.003	40.6	53.1

U, unknown genome location

allele showed smaller average of days-to-flowering than KYU allele, the difference was 8.6 days while KYU allele had early average of days-to-flowering (the difference was 15.3 and 12.5 days, for Fest_L0606_4 and Fest_L0337_6, respectively) in the two QTL's.

To test for the existence of QTL interactions, genotype data for Fest_L0606_4, Fest_L0337_6 and *FeELF3* were used in a two-way ANOVA to test the phenotypic difference among the four homozygous genotype classes (Fig. 5). A significant interaction was detected between Fest_L0606_4 and Fest_L0337_6 ($P < 0.01$). On the other hand, interactions between Fest_L0606_4 and *FeELF3* and between Fest_L0337_6 and *FeELF3*, were not significant ($P = 0.81$ and $P = 0.89$, respectively).

Discussion

A number of studies have been conducted for investigating photoperiod sensitivity in common buckwheat. Minami (1985) suggested that photoperiod sensitivity of common buckwheat is controlled by the action of multiple genes. However, neither genes nor candidate regions related to photoperiod sensitivity had been identified until now. In the present study, a difference in days-to-flowering between the KYU and CAN populations was expressed under a 15.5 h photoperiod, but no difference was observed under the 14.5 h photoperiod. These results showed that the difference in days-to-flowering between KYU and CAN observed under the 15.5 h photoperiod was caused by a difference of photo-

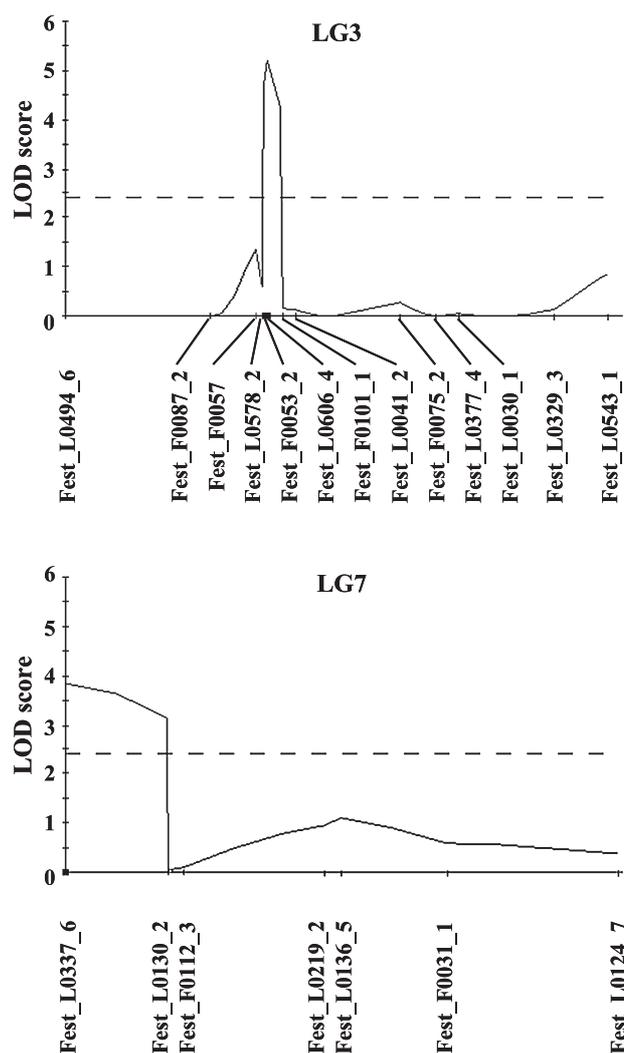


Fig. 4. LOD score for QTLs detected in multiple QTL analysis. The QTL likelihood maps for each linkage group were obtained by using the MQM procedure of MapQTL, fixing one marker cofactor per putative QTL. Selected markers used as cofactors in analysis are indicated by black rectangles along the x-axes. The linkage group number is indicated at the top of each graph. Horizontal dashed lines indicate the 2.4 ($P < 0.05$) LOD score threshold.

period sensitivity: the KYU population had weaker photoperiod sensitivity than the CAN population. The KYU line was bred by interspecific hybridization among *F. homotropicum* (an autogamous species) and *F. esculentum* cv. Botansoba which is cultivated in high-latitude regions (Matsui 2006); similarly, the CAN line was also bred by crossing *F. homotropicum* and *F. esculentum* which is cultivated in high-latitude regions (Wang and Campbell 1998). Thus, we assumed that both lines had been selected under long-day conditions. This may be why the difference in photoperiod sensitivity between KYU and CAN was not evident unless an artificially long day length (15.5 h) was applied.

In *Arabidopsis*, the photoperiod-sensitivity pathway consists of photoreceptors, the circadian clocks, and floral

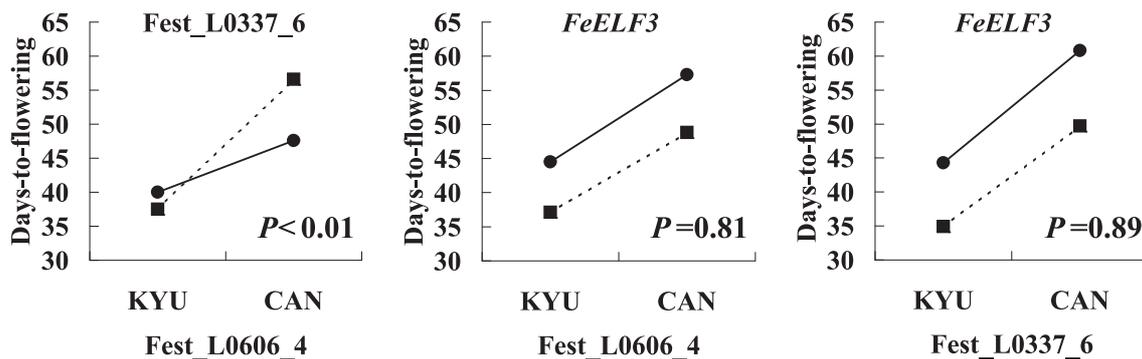


Fig. 5. Mean values for days-to-flowering in different combinations of genotype classes for Fest_L0606_4 and Fest_L0337_6. The x-axis indicates the genotype at Fest_L0606_4 and Fest_L0337_6: KYU and CAN indicate homozygosity for the 02AL113(Kyuhei SC2)LH.self allele and homozygosity for the C0408-0 RP allele, respectively. The genotype of Fest_L0337_6 and *FeELF3* are indicated by circles (homozygous for KYU allele) or squares (homozygous for CAN allele). *P* value in each figure showed results of significance for interaction by two way ANOVA.

promoters (Putterill *et al.* 2004). In many species with photoperiod sensitivity, such as rice, wheat (*Triticum aestivum*), and morning glory (*Pharbitis nil*), genes orthologous to photoperiod-sensitivity genes found in *Arabidopsis* were confirmed to be related to heading date or flowering time (Hayama *et al.* 2002, 2003, Kojima *et al.* 2002, Liu *et al.* 2001a, Nemoto *et al.* 2003, Yano *et al.* 2000). In the present study, we found three such candidate gene regions (*FeCOL3*, *FeCCA1* and *FeELF3*) among 863 cDNA clones. In *Arabidopsis*, *CCA1* and *ELF3* are genes that connect the circadian clock to the photoperiod-sensitivity pathway (Covington *et al.* 2001, Hicks *et al.* 2001, Liu *et al.* 2001b, Wang *et al.* 1997, Wang and Tobin 1998), and *COL3* is from a *CO*-related gene family that connects floral promoters to the photoperiod-sensitivity pathway (Putterill *et al.* 1995). Here, we found that the *FeELF3* candidate gene region in common buckwheat was strongly associated with photoperiod sensitivity. Although the candidate genes identified in the present study would only be part of the total group of genes included in a photoperiod-sensitivity pathway, our report is the first in which candidate genes identified based on their role in photoperiod sensitivity in another species were associated with photoperiod sensitivity in common buckwheat. The results indicate that it would be efficient to study the photoperiod-sensitivity pathway by using other candidate genes and analyzing their effects on photoperiod sensitivity. Although mutations in intron region were reported to be associated with gene expression (Fiume *et al.* 2004, Fu *et al.* 2005, Isshiki *et al.* 1998, Samadder *et al.* 2008), it was not clear whether *FeELF3* marker based on SNP in an intron region directly associated with photoperiod sensitivity right now. If SNP's associated with photoperiod sensitivity in direct will be detected in the future, the allelic variations in *FeELF3* gene region will become more informative information.

In the MQM analysis, we detected two QTLs, Fest_L0606_4 and Fest_L0337_6, which explained about 20.0% and 14.2% the variation observed in the F_4 progenies, respectively. In the present study, we constructed a linkage map for QTL analysis consisting of 9 linkage groups con-

taining 63 ESTs and two candidate gene regions and covering 311.6 cM in total. This map was the first linkage map to include ESTs and candidate gene regions thought to be related to photoperiod sensitivity in common buckwheat. Unfortunately, we assumed that the genome coverage of this map is not so high because Konishi and Ohnishi (2006) constructed a linkage map of buckwheat covering about 900 cM by using AFLP and SSR markers. Thus, result of MQM analysis at unmapped *FeELF3* candidate gene region was not shown. If the genome coverage of this map will be higher, other QTLs included *FeELF3* candidate gene region might be detected.

The genetic analysis of quantitative traits with molecular markers makes it possible to evaluate epistatic interactions between individual loci. A digenic interaction between the QTLs detected in the MQM analysis was observed in this study. The additive effect of the KYU allele of Fest_L0337_6 was estimated to be -6.8 days. However, the observed two-locus interaction between Fest_L0606_4 and Fest_L0337_6 suggests that the effects of Fest_L0337_6 are changed depending on the allele present at Fest_L0606_4. In *Arabidopsis* and rice, interactions between genes related to photoperiod sensitivity have been reported (Putterill *et al.* 2004, Yano *et al.* 1997). These results suggested that photoperiod sensitivity in common buckwheat was controlled by both main and interaction effects of the QTLs detected. Thus, it is necessary to analyze both the main effects and the interactions between QTLs to fully understand photoperiod sensitivity.

In summary, our study is the first report that photoperiod sensitivity in common buckwheat is controlled by the action of multiple genes, including interaction effects between these genes. The photoperiod sensitivity was controlled by at least three loci, including candidate genes identified as being part of the photoperiod-sensitivity pathway in *Arabidopsis*. These findings will serve as a stepping-stone to future studies on the role of photoperiod sensitivity in local adaptation and ecological breeding in common buckwheat.

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