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Identification of RFLP markers linked with heading date and its heterosis in hexaploid wheat

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Summary

Recombinant inbred lines (RILs) from a cross between hexaploid wheat (*T. aestivum* cv. Chinese Spring (CS) and *T. spelta* (Sp)) were used for RFLP analysis of heading date and heterosis. Fourteen RFLP markers linking with heading date were identified; two were localized on chromosome 1A, one on 2A, three on 2B, one on 2D, four on 5A, two on 7A and one unlinked but reported to be on group 2. All of these markers may be attributable to genes for earliness *per se*. However, the markers in the chromosomes of 1A and 7A are new to this study. RILs were crossed with (*tim*)-CS, the alloplasmic CS with *T. timopheevi* cytoplasm, and the heterosis from earlier-parent and mid-parents were calculated for the F_1s to examine the heterotic effect toward earliness on heading date. Five and two RFLP markers were associated with heterosis from the earlier-parent and mid-parents, respectively. They were distributed on the chromosomes of homoeologous groups 1 and 2.

Introduction

Heading date is a critical determinant of crop adaptation. Many genes are involved in fitting a crop cultivar to the prevailing environmental conditions; for example photoperiod and vernalization response genes that cause plants to change from vegetative growth to reproductive growth. Also, heading date is influenced by genes independent of environmental conditions (earliness *per se*) (Kato & Wada, 1999).

Wheat cultivars are usually classified into two growth habits, namely, spring and winter types. The major physiological determinant of the difference between the two types is the requirement of winter wheats for a certain period of growth at low temperature, called vernalization, before floral initiation.

In hexaploid wheat, there are five genetic loci involved in response to venalization requirement and the chromosomal locations of four have been identified; namely *Vrn-A1* on 5A (formerly *Vrn1*), *Vrn-D1* on 5D (formerly *Vrn3*), *Vrn-B1* on 5B (formerly *Vrn2* or *Vrn4*) and *Vrn-B4* on 7B (formerly *Vrn5*) (Law et al., 1976; Snape et al., 1985; Galiba et al., 1995; Kato et al., 1998; Snape et al., 1998). Winter wheat cultivars carry recessive alleles at each of these loci. *Vrn-D1* is common in certain semi-spring varieties such as Chinese Spring, although its potency in requiring vernalization appears less than *Vrn-A1*. The intra-chromosomal locations of *Vrn-A1* and *Vrn-D1* have been identified using molecular markers (Galiba et al., 1995; Snape et al., 1998).

Genes responding to photoperiod also affect heading date. They are designated as *Ppd-D1* (formerly *Ppd1*), *Ppd-B1* (formerly *Ppd2*), and *Ppd-A1* (formerly *Ppd3*) on the short arms of chromosomes 2D, 2B, and 2A, respectively (Pirasteh & Welsh, 1975; Scarth & Law, 1983). The dominant alleles at the *Ppd* loci confer insensitivity to photoperiod.

This study was aimed at defining the genetic system for heading date determination of common wheat. By analyzing a population of recombinant inbred lines by RFLP markers, QTLs could be detected for heading date. Furthermore, we identified loci showing het-

erotic effects on the heading date using F_1 hybrids between the RILs and one of the parental cultivars.

Materials and methods

Plant materials

The F_8 population of recombinant inbred lines (RILs) derived from a cross between *Triticum aestivum* cv. Chinese Spring (CS) and *T. spelta* var. *duhamelianum* (Sp) was used for this investigation (Sasakuma & Tsujimoto, Unpub.), where CS is an early parent and Sp is late. CS is a semi-spring type of wheat having *Vrn-D1* and *Ppd-B1* that show medium requirement for vernalization and photoperoid, whereas Sp is a spring type that does not require vernalization because of possessing *Vrn-A1*.

Sixty-six RILs in addition to the parents (CS and Sp) were grown in the experimental field of Kihara Institute, Yokohama, Japan, in a randomized complete block design (RCBD) with two replications. Seeds were sown first in nursery boxes in November, and were transplanted to the field in December in the seasons of 1995/6 and 1997/8. The temperature of winter in Yokohama is below 10 °C on average for more than two months, so that plants should be completely vernalized. Heading was observed in three plants for each replication.

For the analysis of heterosis, each RIL was crossed as male to an alloplasmic line of CS with *T. timopheevi* cytoplasm (abbrev. (*tim*)-CS). The cytoplasm of *T. timopheevi* induces complete male sterility on the CS genetic background, yet does not affect the heading date (Tsunewaki et al., 1980). The F_1's were grown in paired comparisons with the corresponding RILs in 1998.

Evaluation of heading date and heterosis

Heading date was defined as the number of days from the first of April to the date when the first spike had completely emerged. Analysis of variance (ANOVA) for each year and for the combined years was carried out. The differences between the means of lines were tested by the least significant differences test (LSD). Mean squares (MS) obtained from ANOVA were partitioned into expected mean squares (EMS) to calculate genotypic and phenotypic variances. Heritability (h^2) was calculated from the following formula:

$$h^2 = \sigma^2 g/(\sigma^2 g + \sigma^2 e),$$

where $\sigma^2 g$ is the genotypic variance and $\sigma^2 e$ is the environmental variance.

The heterosis for heading means the phenomena that any F_1 lines headed earlier or later than their respective parents or the earlier parent. Heterosis from the mid-parent and the earlier-parent was estimated for heading date from the following equations, [1] and [2], respectively:

$$\text{Mid-parent heterosis (\%)} = 100 \times (F_1 i - \text{MP}i)/\text{MP}i \quad [1]$$

$$\text{Earlier-parent heterosis (\%)} = 100 \times (F_1 i - \text{EP}i)/\text{EP}i, \quad [2]$$

where $F_1 i$ is the heading date of the respective F_1 obtained from the cross between CS and RIL no.*i*, MP*i* is the average heading date between CS and RIL no.*i*, and EP*i* is the heading date of earlier parent of either CS or RIL no.*i*.

DNA hybridization and RFLP analysis

DNA was isolated from fresh leaf tissues of each RIL and the parents. For each sample 10 μg of total DNA was digested with nine restriction enzymes, *viz*. *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Bam*HI, *Xba*I, and *Xho*I, to screen for polymorphism between CS and Sp. Restricted DNAs were electrophoretically fractionated in 0.85% agarose gels and transferred to Hybond N^+ nylon membranes (Amersham) by capillary transfer utilizing 0.4 N NaOH.

Two hundred-thirty one DNA markers of genomic and cDNA clones of wheat, barely, oat, and rice were used for polymorphism screening [PSR (Gale et al., 1995), KSU (Gill et al., 1991), TAG (Liu & Tsunewaki, 1991), ABC (Kleinhofs et al., 1988), BCD, CDO, WG, and RZ (Anderson et al., 1992), and CS (Shindo & Sasakuma, submitted)]. DNA inserts were isolated from plasmids either by restriction enzyme digestion, electrophoresis and elution by centrifugation, or by PCR amplification using plasmid primers. Probes were labeled with α-^{32}P-dCTP by the random hexamer primer method (*Bca*BEST, Takara). Hybridization was carried out at 65 °C for 24h in a rotary hybridization chamber (Hybaid). The membranes were washed in 2 × SSC, 0.1% SDS for 15 min at 65 °C, and 0.5 × SSC, 0.1% SDS twice for 20 min each at 65 °C, and then rinsed with 2 × SSC for about 2 min at room temperature. Hybridization blots were exposed to X-ray BioMax film (Kodak), with intensifying screen at –80 °C for 4–5 days, depending on the intensity of signals.

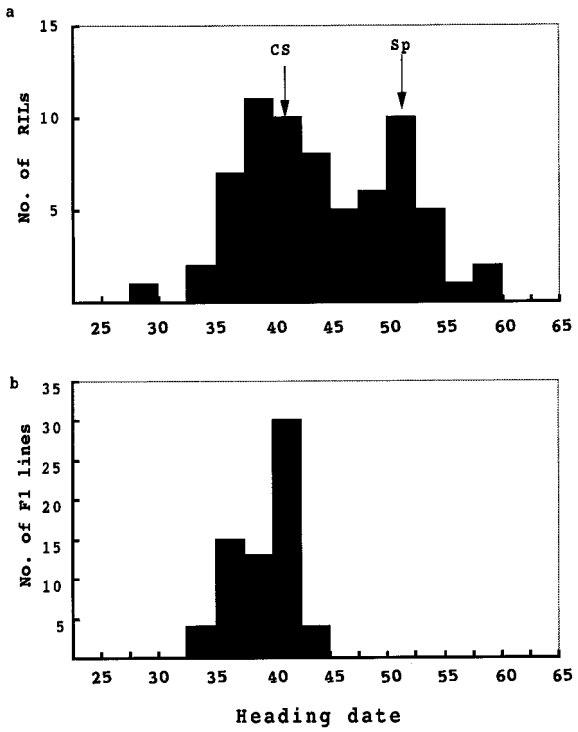

Figure 1. Distribution of heading date in 1998, in RIL population (a) and in hybrids population (b). The dates of CS and Sp are indicated by arrows. These data were obtained in 1998.

Table 1. Analysis of variance of heading date over two years and expected mean squares (EMS)

SOV	df	MS	EMS
Year	1	2.5	
Rep/year	2	3.7	
Lines	67	164.9**	σ^2e+rσ^2yl+ryσ^2l
Lines × Year	67	14.9**	σ^2e+rσ^2yl
Error	134	3.9	σ^2e
Total	271		

** Significant at the 1% level. σ^2e, σ^2l, σ^2yl, y, and r are environmental variance, genotypic variance, year × line variance, years, and replications, respectively.

Linkage analysis

One hundred of the polymorphic RFLP probes showed 151 marker loci. The loci were tested on the 66 RILs of CS and Sp. Linkage analysis of the loci was performed with a computer program, MAPMAKER ver. 2.0 for Macintosh (Lander et al., 1987). We used the mapping function of Kosambi (Kosambi, 1944). The linkage groups were assigned to the wheat chromosomes according to the published wheat maps (GrainGene database) and RFLP patterns of nulli-tetrasomic lines of Chinese Spring. The LOD threshold used was 3.0 and lowered to 2.5 to add unlinked markers to the linkage groups.

QTL analysis

The QTL analysis was performed as a single marker analysis using the QGENE computer program (Nelson, 1997). This program was used to determine the association between individual marker loci and putative QTLs. This program uses individual marker-genotype groups as class variables for detection of linkage between markers and putative QTLs. Associations of marker loci with QTLs were considered to be significant when the F-test exceeded a value necessary for a probability value less than 0.02. This significance level was chosen to maximize the identification of putative QTLs. Single locus R^2 values were calculated for each significant marker locus. The phenotypic effect was calculated as the difference between the classes means of CS and Sp.

Results

Heading date of RIL

The mean heading dates over two years ranged from 27.3 (RIL no. 65) to 55.8 (RIL no. 1) (Figure 1a). The analysis of variance revealed significant differences ($p < 0.01$) among RILs in both seasons as well as the combined data (Table 1). LSD indicated that seven lines headed earlier than CS, and 37 lines were later. On the other hand, 54 lines headed earlier than Sp, but no lines were significantly later.

The heritability in 1998 was slightly higher than that in 1996. However, in both years the genotypic variance, which contributes to the increase in heritability, was much higher than the environmental variance (Table 2).

Heterosis

The analysis of variance for the F_1 population showed significant differences ($p < 0.01$) among the means. In general, F_1 lines tended to flower earlier than their respective RILs (Figure 1b). The heading date of F_1s ranged from 33.3–44.2, whereas in RILs it ranged from 28.8–58.5. The variance of F_1s (7.1) was less

Table 2. Means, variance components, and broad-sense heritability (h^2) for heading date

	1996	1998	Combined
Mean			
CS	40.0	40.0	40.0
Sp	51.8	56.2	54.0
RILs	44.8	44.6	44.7
Variance[1]			
σ^2g	40.3	45.8	37.5
σ^2p	46.5	47.2	41.4
h^2	86.6	97.0	90.6

[1] σ^2g, σ^2p indicate genotypic and phenotypic variance, respectively.

than that of RILs (48.8). Deviation of F_1 performance from its respective RIL was analyzed for each pair individually by use of the t-test. Forty-five F_1 lines flowered significantly earlier than their respective RILs, whereas three F_1 lines were significantly later than their RIL parents.

The analysis of variance showed significant differences between means of both mid-parent heterosis and high-parent heterosis. In mid-parent heterosis, the most positive heterosis (heterosis for earliness) was observed in the F_1 of no. 63 (14.05%); whereas the most negative heterosis (heterosis for lateness) was in the F_1 of no. 60 (–7.22%). On the other hand, in high-parent heterosis, the most positive heterosis was in the F_1 of no. 16 (12.30%), and the most negative was in the F_1 of no. 65 (–19.10%).

Linkage analysis with RFLP markers

Of the 231 RFLP probes used, 100 (43.3%) showed a polymorphism between the parental lines with at least one enzyme. These probes revealed 151 marker loci, which were employed for map construction, and to check the linkage between the markers and the phenotype of heading date in 1996 and 1998, as well as heterosis (Figure 2).

Five RFLP markers linking with heading date were detected in both years, and an additional three, and six exhibited only in 1996 and 1998, respectively (Table 3, Figure 2). Two of the markers were located on chromosome 1A, one on 2A, three on 2B, one on 2D, four on 5A, two on 7A, and one unlinked but it is reported to be located on group 2. Markers of six CS alleles (psr103, psr135, cs14-1, psr131c, psr131a, and psr126b), and eight Sp alleles (rz630, ksuF11b, cdo1160, cdo412, psr128a, psr574, psr899c, and ksuD30b) showed earliness (Table 3).

Seven markers were linked with heterosis data (Table 4). Five of the markers were linked with earlier-parent heterosis, three of which showed linkage with those of heading date on chromosomes 2A and 2B. The chromosome location of the other marker could not be identified. Two markers showed linkage only with mid-parent heterosis. No QTLs could be identified with both types of heterosis together.

Discussion

Analysis of heading date

Many researchers studied the photoperiod and vernalization-response genes by F_2 segregation or aneuploid analysis (Law et al., 1976; Snape et al., 1985; Kato & Yamashita, 1991; Galiba et al., 1995; Snape et al., 1997). In the present RIL population of hexaploid wheat the bimodal distribution of heading dates showed inheritance with one major gene, although we know in our population that at least *Vrn-A1*, *Vrn-D1*, and *Ppd-B1* are segregating. Under the field condition of this experiment, we should say that the plants would have been completely vernalized by the low temperature in winter. Thus, this distribution may be mainly due to the *Ppd-B1* on chromosome 2B.

The heritabilities for heading date were high in both seasons and in the combined seasons. These results are in agreement with Law (1966), who reported that ear emergence in wheat as a quantitative character has a high heritability, and that the identification of factors responsible for the character may not be as difficult as that for factors involved in other quantitative characters with smaller heritabilities. However, in the present study we analyzed the genes in more detail using molecular markers, because the genetic backgrounds of the two parental lines were highly different.

In this study we identified 14 RFLP markers linked with genes affecting heading date on chromosomes of homoeologous groups 1, 2, 5, and 7. Although the analysis of variance did not show significant difference between the two seasons in heading date, RILs responded differently to years, where Year × RIL interaction was significant (Table 1). Five markers (psr103, cdo1160, psr131a, psr131c, and psr135) linked with heading in both 1996 and 1998. Three markers (rz630, cs14-1, and ksuF11b), and six markers (psr 126b, psr128a, psr574, psr899c, psr128b, and

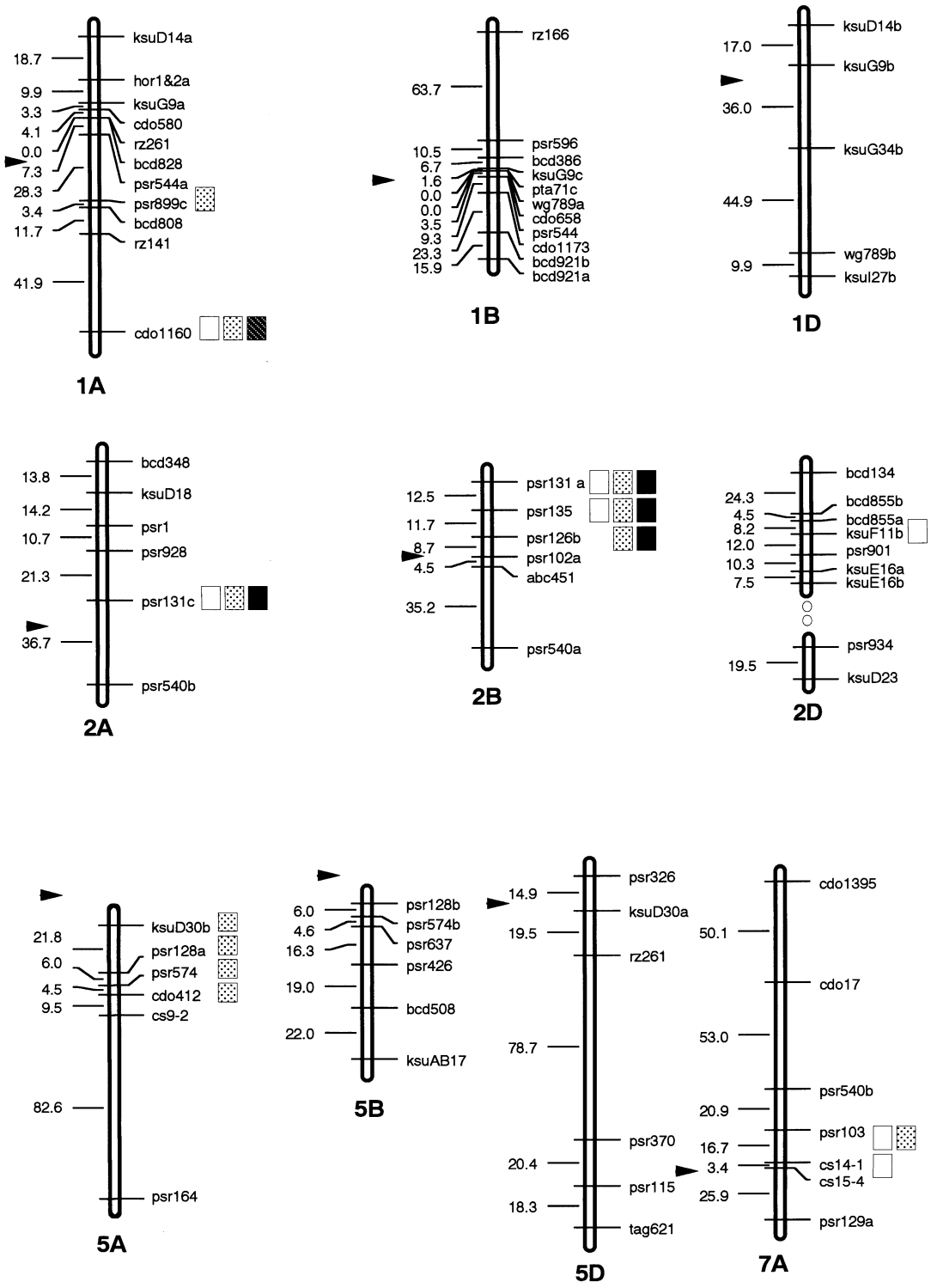

Figure 2. An RFLP linkage map of wheat chromosomes (group 1, 2, 5, and 7A) constructed based on a RIL population of a cross between *T. aestivum* (CS) and *T. spelta* (Sp). The names of the markers and their map positions (in cM based on Kosambi function) are shown at the left and the right of the chromosomes, respectively. The approximate position of the centromers, indicated by *arrows*, were deduced from published wheat maps. Boxes which are opened, with dots, shaded, and black, in front of the markers, indicate the QTLs detected for heading date of 1996, 1998, mid-parents heterosis, and earlier-parent heterosis, respectively.

Table 3. QTLs detected for heading date for 1996, 1998 seasons based on single marker analysis

Marker name	Chromosome	F-value	Probability	R^2 %	Allele*		Phenotypic effect[a]
					CS	SP	
Heading 1996							
psr103	7A	15.3	0.0002	20.9	41.9	47.9	–6.0
psr131c	2A	10.5	0.0019	14.1	41.5	46.6	–5.1
rz630	–	9.6	0.0029	13.2	47.6	42.8	4.8
psr135	2B	8.1	0.0064	13.5	42.4	47.0	–4.6
cs14-1	7A	6.6	0.0130	9.8	42.4	46.5	–4.1
ksuF11b	2D	6.3	0.0149	9.2	46.3	42.2	4.1
cdo1160	1A	6.0	0.0170	9.1	46.7	42.8	3.9
psr131a	2B	5.8	0.0188	8.7	42.8	46.6	–3.8
Heading 1998							
psr131c	2A	12.7	0.0007	16.6	41.0	46.7	–5.7
psr103	7A	9.8	0.0027	14.5	42.1	47.3	–5.2
psr126b	2B	9.6	0.0029	13.0	42.2	47.1	–4.9
psr131	2B	9.5	0.0032	13.4	42.1	47.1	–5.0
psr135	2B	8.2	0.0060	13.7	42.0	47.0	–5.0
cdo412	5A	7.8	0.0066	12.5	46.7	41.7	5.0
psr128a	5A	7.3	0.0089	10.4	46.3	41.9	4.4
psr574	5A	7.2	0.0094	10.4	46.8	42.4	4.4
psr899c	1A	7.1	0.0099	10.1	46.4	42.2	4.2
cdo1160	1A	6.8	0.0114	9.9	46.8	42.5	4.3
ksuD30b	5A	6.7	0.0120	10.1	46.7	42.4	4.3

–; means the marker unlinked to the map.
*; means the heading date means of the sub-populations of CS type and Sp type.
[a] Phenotypic effect = difference between the heading date of means of CS and Sp sub-populations.

Table 4. QTLs detected for heterosis of heading date for mid-parents and early-parent based on single marker analysis

Marker name	Chromosome	F-value	Probability	R^2 %	Allele*		Phenotypic effect[a]
					CS/CS	CS/Sp	
Heterosis of mid-parents							
ksuD18a	–	9.8	0.0027	14.0	10.4	6.8	3.6
cdo1160	1A	7.9	0.0068	11.2	7.4	10.5	–3.1
Heterosis of earlier-parent							
psr131c	2A	18.2	0.0001	22.1	12.1	17.9	–5.8
psr126a	–	10.2	0.0022	14.1	13.2	17.7	–4.5
psr126b	2B	8.7	0.0044	12.0	13.7	17.9	–4.2
psr131a	2B	7.4	0.0085	10.8	13.6	17.6	–4.0
psr135	2B	7.2	0.0099	12.1	14.2	18.0	–3.8

–; means the marker unlinked to the map.
*; means the heterosis mean of the sub-population of CS/CS type and CS/Sp type.
[a] Phenotypic effect = difference between the heterosis percentage of CS/CS and CS/Sp sub-populations.

ksuD30b) linked with heading in 1996, and 1998, respectively (Figure 2, Table 3). These findings indicate that these linked loci are environmentally sensitive ones.

Five markers linked with heading on group 2 chromosomes. The three markers associated with QTL on chromosome 2B (Figure 2) linked with each other at 12.5, and 11.7 cM, suggesting that a gene affecting heading date is located near these markers. For this QTL, the allele of CS promoted heading early (Table 3). Since the psr131a and psr131c are homoeologous loci, the QTLs on chromosomes 2A and 2B must be in homoeologous relationship as well. The chromosomes of homoeologous group 2 are important in determining the time of ear emergence under different day lengths (Law et al., 1978). Keim et al. (1973) defined two major genes controlling day-length response: *Ppd1* (*Ppd-D1*) on chromosome 2D, which is dominant and epistatic for insensitivity, and *Ppd2* (*Ppd-B1*) on chromosome 2B, which is dominant for partial insensitivity. Scarth & Law (1983) found photoperiod-response gene *Ppd2* (*Ppd-B1*) on chromosome 2B. In addtion, it was reported that genes for earliness *per se* associated with RFLP markers near the centromere of group 2 chromosomes (Worland, 1996).

Psr126b, psr135 and psr131 are reported to be located at 2.9, 3.9 and 12.8 cM, respectively from the centromere on the short arm of chromosome 2B (Gale et al. 1995). Worland (1996) reported that genes for earliness *per se* associated with the group of centromeric RFLP markers, indicating that those genes must be located in this region. From this result and the distribution of heading, it would be suggested that these three markers with the CS alleles identified in the present experiment should be linked with earliness *per se*. The QTL of Sp on chromosome 2D associated with ksuF11b may be homoeologous to that on chromosome 2B, because their locations are located near the centromere.

The Sp allele of five RFLP markers (cdo412, psr128a, psr128b, psr574, and ksuD30b) on chromosome 5A promoted ear emergence (Table 3). However, our preliminary study showed that these markers were not linked with *Vrn-A1* of this chromosome (Shindo et al., in preparation). In fact, these markers were reported to be mapped apart from *Vrn-A1*. These facts indicate that the QTL is related to earliness *per se*. Many researchers explained the importance of group 5 chromosomes as a carrier of vernalization response genes. CS carries *Vrn-D1* on chromosome 5D, and Sp carries *Vrn-A1* on chromosome 5A (Galiba et al., 1995; Snape et al., 1997). These genes are considered to be important in determining heading date when vernalization is incomplete. Under the conditions of the present field experiment, the plants would have been completely vernalized by the low temperature during winter. Thus, we should consider other genes affecting heading date on chromosomes of homoeologous group 5.

There were two RFLP markers on chromosome 1A linked with heading date (Table 3), indicating that these chromosomes also carries genes affecting heading date, though no genes responding to temperature or day length were reported. These genes, therefore, play an important role in determination of earliness *per se*. Law et al. (1998) reported that in CS each of the homoeologous group 1 chromosomes delayed emergence. They found that the genes on chromosome 1A were the most potent. Our study extends their results to the DNA marker level. The CS allele of psr899c on chromosome 1A was associated with later heading (Table 3).

Psr103 on chromosome 7A showed a strong QTL ($p = 0.0002$) for heading, showing gene(s) affecting heading on 7A. In barley, it was reported that flowering time was affected by genes on chromosome 7H whose effects were not specifically dependent on photoperiod or vernalization (Laurie et al., 1995). Consequently, this QTL may be homoeologous to the QTL of barley for earliness *per se*, though synteny between barley and wheat chromosomal regions around the QTLs could not investigated because of lack of common RFLP markers in both of the species.

The identified QTLs had the favorable alleles (alleles of earliness) from both parents. This finding demonstrates the ability of marker analysis to uncover cryptic genetic variation that otherwise would have been masked by the large difference between the parents. The presence of favorable QTL alleles in both parents suggests a strong likelihood for recovering transgressive segregants and provides a source of new alleles for plant breeding.

Heterosis

Hybrid wheat production with *T. timopheevi* cytoplasm has been studied for several decades (Briggle, 1963; Knott, 1965; Sun & Wang, 1985; Wang, 1985; Wang et al., 1985). The system utilizing this cytoplasm has been largely used because the result of its apparently neutral effect on agronomic characters

(Tahir, 1970; Tsunewaki et al., 1980; Liu & Li, 1994). Also, in the present study no significant difference in heading date between (*tim*)-CS and CS was observed. We employed this CMS line for the convenience of producing respective F_1's with each RIL.

In the present study, most of the F_1 hybrids between (*tim*)-CS and each RIL displayed earlier heading than their respective RILs. This is due to the genetic background of earlier heading in CS than in Sp, and both *Vrn* and *Ppd* genes would be dominant. The hybrids in these crosses have entire set of CS genes in one half and the respective RIL genetic components in the other half. Therefore, varying proportion of each hybrid were homozygous for CS alleles, whereas the remaining portions were heterozygous. Some hybrids showed high heterosis towards earliness, whereas others showed no heterosis.

Two linked markers (cdo1160 and ksuD18a) one on chromosome 1A, and one unlinked, were associated with heterosis from the mid-parent (Table 4). However, these markers were not involved with either high-parent heterosis or the heading date itself. One of the two markers (cdo1160 on 1A) was the Sp type (Table 4), clearly indicating that there is a gene that promotes heterosis only when it is present in the heterozygous state.

Among the five RFLP markers detected for earlier-parent heterosis, four were also involved directly in heading date (Table 4). When they were analyzed for genotype, all the four were heterozygous, conferring allelic heterosis or dominance (Table 4). The QTL linking with remaining one marker unidentified on group 2 chromosomes (psr126a) exhibited earlier-parent heterosis in heterozygous state, indicating that this QTL is a promoter elements only for heterosis.

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