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Molecular mapping of major genes influencing flowering time in wheat (*Triticum aestivum* L. em. Thell)

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1 Introduction

Wheat is one of the world's most important food grain. It provides over 20 % of calories and protein in human nutrition, and is the staple food in more than 40 countries for over 35 % of the world's population. It is the most widely cultivated food crop, from the southern regions of South America and Australia to the northern latitudes of Canada and China, and can grow over a wide range of elevations, climatic conditions and soil fertility (Bushuk, 1998).

Part of this wide adaptability is due to the exploitation of genes that control ear-emergence time. The most important of these are the genes for vernalisation and photoperiodic responses which are associated with the geographical origins of different wheat varieties (Miura and Worland, 1994). A main objective of wheat breeders is to match genotypes to environments to reach the maximum yield stability across location and years (Ferrara et al., 1998). This effort allows a better targeting of germplasm to specific environments, reduces the risk of crop failure, helps in the development of more realistic crop models, and insures input by control optimum production.

In recent years, significant progress has been made in the use of molecular approaches for plant breeding. Molecular marker closely linked to numerous traits of economic importance have been developed which will allow indirect selection for desirable traits in early segregating generations at the seedling stage. This will save time, resources and energy that are needed not only for raising large segregating population for several generations, but also for estimating the parameter used for direct selection (Caetano-Anolles & Gresshoff, 1997). Furthermore, the indirect selection is very effective due to the absence of confounding effect of environment, and also allows pyramiding of genes for characters like disease resistance, which is difficult through the use of conventional methods of plant breeding (Gupta and Varshney, 2000).

In this research, molecular markers linked to major photoperiod (*Ppd-B1*) and major vernalisation (*Vrn-D1)* genes in wheat were searched using AFLP and SSR techniques. The use of different populations carrying the same gene enabled to answer the crucial question in MAS whether the markers found in certain cross can be also applied in different crosses. Furthermore a simple method for scoring the presence of *Ppd-B1* gene was also developed by conversion the AFLP markers to sequence-specific PCR markers.

1.1 Genetics of flowering time in wheat

The major components of the life cycle are the time between sowing and emergence, the period after emergence when the crop grows vegetatively before the onset of floral initiation, the length of the period of floral initiation to terminal spikelet, the period between terminal spikelet and heading, and finally, the time of flowering through grain filling to maturity. Although the timing of sowing is environmentally determined, most of the other components vary between different varieties, and hence, are under genetic control (Snape et al., 2001a).

The life cycle of wheat is determined predominantly by three sets of genes: a) Vernalisation (*Vrn*) and b) photoperiod (*Ppd*) genes act in response to the environmental stimuli cold and day length, whilst c) earliness per se (*Eps*) genes act independently of the environment, determining the number of vegetative and floral primordia being initiated. The three sets of genes influencing flowering time act together to determine the exact time of flowering and hence the suitability of a genotype for flowering under particular environmental conditions (Worland, 1996; Worland et al., 1998). Through studies using a wide array of precise genetic stocks and techniques of genetic analysis, the complex genetic control of flowering time has been elucidated. It is likely that virtually all 21 pairs of hexaploid wheat chromosomes carry genes that influence flowering time (Table 1).

Table 1. A literature review of chromosomal and genetical effects determining flowering time in wheat (Worland, 1996)

Group	
1	Carry genes for sensitivity to vernalisation (Scarth & Law, 1983)
2	Major photoperiod insensitive genes *Ppd1*, *Ppd2* and *Ppd3* on the short arms of chromosomes 2D, 2B and 2A respectively (Welsh et al., 1973; Scarth & Law, 1983; Sharp & Solltes-Rak, 1988). Earliness *per se* genes on long arms of 2B (Scarth and Law, 1983) and 2D (Worland, unpublished).
3	Chromosome 3A carries gene(s) for earliness *per se* (Hoogendoorn, 1985; Miura & Worland, 1994). Chromosome 3B carries gene(s) for vernalization response (Kosner, 1987; Miura & Worland, 1994). Chromosome 3D carries a gene for photoperiod insensitivity (Miura & Worland, 1994).
4	Chromosome 4A* (Nomenclature post 7th Int. Wheat Genet. Symp. 1988) and 4D promote earliness *per se* (Hoogendoorn, 1985).
5	Major vernalization insensitive genes *Vrn1*, *Vrn2* and *Vrn3* on the long arms of chromosomes 5A, 5B and 5D respectively (Law et al., 1976, Maistrenko, 1980). Determine spring/winter differences.
6	Homoeologous group 6 chromosomes carry genes that reduce the need for vernalization (Faridi,1988) Chromosomes 6B carries genes for photoperiod sensitivity (Faridi, 1988). Chromosome 6B and 6D promote earliness *per se* (Hoogendoorn, 1985; Law, 1987).
7	Chromosome 7B carries the vernalization insensitive gene *Vrn5* (Law and Wolfe, 1966). Similar vernalization effects detected on chromosome 7A (Faridi, 1988). Earliness *per se* effets on chromosome 7B (Flood and Halloran, 1983; Hoogendoorn, 1985).

1.1.1 Genetic control of vernalisation response

Wheat can be classified into winter types, which require vernalisation to promote flowering, and spring types which do not. Major genes controlling sensitivity to vernalisation, the *vrn* genes, determine the control of spring wheat/ winter wheat difference. The lack of vernalisation requirement is generally dominant. Substitution line analysis has identified four loci, namely *Vrn-A1* on chromosome 5A (formerly *Vrn1*), *Vrn-D1* on chromosome 5D (formerly *Vrn3*), *Vrn-B1* on chromosome 5B (formerly *Vrn2*) and *Vrn-B4* on chromosome 7B (Table 2).

Table 2. Major *Vrn* genes and their location in wheat

Vrn genes	Former name	Location on Chromosome	References
Vrn-A1	*Vrn1*	5A	Law et al., 1976; Snape et al., 1985
Vrn-B1	*Vrn2*	5B	Gotoh, 1979; Iwaki et al., 2001
Vrn-D1	*Vrn3*	5D	Law et al., 1976, 1980
Vrn-B4	*Vrn4*	7B	Gotoh, 1979; Iwaki et al., 2001

Vrn-A1 is the most potent gene which gives insensitivity to vernalisation, and is epistatic to the other three genes (Pugsley, 1971). On the other hand, *Vrn-B1*, *Vrn-D1* and *Vrn-B4* give weak sensitivity to vernalisation (Pugsley, 1971, 1972) and no epistatic relationship has been reported among them. Relative frequencies of *Vrn* genes in commercial varieties were determined as *Vrn-A1*, 80 %; *Vrn-B1*, 64 %; *Vrn-D1*, 18 %; *Vrn4* and *Vrn5*, 0 % of varieties (Gotoh, 1979; Rigin et al., 1985; Stelmakh et al., 1987). Stelmakh (1990) surveyed *Vrn* genotype of improved cultivars from Europe and Asia, and showed that *Vrn-A1* was frequent in Europe and Siberia. On the contrary, *Vrn-D1* was frequently found in Asia, indicating that, even within spring wheat, cultivars with different *Vrn* genotype adapted to different growing conditions.

A more detail survey about eco-geographical differentiation in East Asia (China, Korea and Japan) has been carried out recently by Iwaki et al. (2000). They found that spring type landraces accounted for 43.6 % of the whole, and the frequency varied between the localities, being closely related to the degree of winter coldness. Spring type landraces mainly adapted to North and South China where average January temperature from –7°C to 4°C. Genetic analysis for spring type landraces showed that the relative frequency of four *Vrn* genes was different with each other. In North China, many landraces proved to carry *Vrn-D1* along with another *Vrn* gene(s), and thus the frequency of *Vrn-A1* was the highest (76.9 %), followed by *Vrn-B1* (50 %) and *Vrn-D1* (50 %). On the other hand, most of the landraces carried *Vrn-D1*

as a single dominant gene in South China and Southwest Japan, and carrier of *Vrn-A1* existed sporadically. Such a differential distribution of *Vrn* genes clearly indicated that geographical variation of *Vrn* genotypes closely related with the degree of winter coldness.

The effect of the *Vrn-A1* gene on floral development has been examined by studying the phenology of a *Vrn-A1* isogenic line in the genetic background of the UK winter wheat, Hobbit 'Sib' (Snape et al., 2001a). This isogenic contained the *Vrn-A1* allele from a spring line of *Triticum spelta*, which is strongly vernalisation insensitive. Several sowings were carried out in the autumn through to spring, and plants sampled for stage of development from sowing through the winter until flowering in the spring. The presence of the spring allele resulted in a faster rate of development, with both vegetative and reproductive primordia being produced at a quicker rate. This difference is maintained during and after vernalisation, and results in an earlier flowering even following complete vernalisation. This difference is attenuated in early spring sowings where vernalisation treatments are lower, and thus flowering times of the vernalisation sensitive line much later.

Similar experiment was also carried out by Law et al. (1980) using single chromosome recombinant lines developed between Chinese Spring and an intervarietal chromosome substitution line in which chromosome 5D of Chinese Spring was replaced by its homologue from the variety Hope. In this material recombination is restricted to chromosome 5D on which Chinese Spring carries the vernalisation insensitive *Vrn-D1* allele and Hope the sensitive *vrn-D1* allele. The single chromosome recombinant lines were classified for the presence of *Vrn-D1* or *vrn-D1* and grown in replicated field trials from late autumn, early spring and late spring sowings. From an autumn sowing all lines flowered at a similar time as cool conditions met after germination delayed growth of all genotypes and satisfied the vernalisation requirements of *vrn-D1*. Thus when temperatures increased in spring and growth resumed both genotypes could initiate floral primordia and run up to flowering at similar times. From an early spring sowing development of vernalisation sensitive genotypes was delayed until vernalisation requirements were satisfied resulting in a 14.2 day delay in flowering over insensitive lines. From the later spring sowing where temperatures were warmer and nightly period of cooler temperature became reduced the flowering time difference between vernalisation sensitive and insensitive genotypes increased to 28.1 days. An even later sowing could result in an inability of the vernalisation sensitive genotypes to flower if warmer temperatures prevented vernalisation requirements being satisfied. These results could be interpreted as that the presence of a 'spring' allele stimulates the production of a substance which stimulates primordia production at a faster rate than the sensitive allele

which only induces production of such a substance following vernalisation (Snape et al., 2001a).

Intensive chromosome mapping has been carried out mainly by using restriction fragment length polymorphisms (RFLPs), and several RFLP loci linked to *Vrn* genes have been reported. The *Vrn-A1* and *Fr1* loci are closely linked on the distal portion of the long arm of chromosome 5A, with a map distance of 2 cM. The RFLP marker loci *Xpsr426* and *Xwg644* are tightly linked to the *Vrn-A1* locus (Galiba et al., 1995). The intrachromosomal location of *Vrn-D1* and *Fr2* on chromosome 5D was also established recently using RFLP, amplified fragment length polymorphism (AFLP) and microsatellite markers (Snape et al., 1997, 1998). The study showed that *Vrn-D1* and *Fr2* are linked on the long arm of 5D and that *Fr2* appears to be further away, genetically, from *Vrn-D1*, than *Vrn-A1* is from *Fr1* (Snape et al., 1998).

Using wheat chromosome deletion lines which were generated in the variety 'Chinese Spring', Sutka et al. (1999) found that the *Vrn-A1* gene mapped between break-points 0.68 and 0.78, whilst the frost resistance gene *Fr1* was flanked by deletion breakpoints 0.67 and 0.68. This confirms previous evidence that the expression of these traits are controlled by individual genes and are not the pleiotropic effect of a single gene.

Vrn-D1 was located, as expected, distally on the long arm of 5D, closely linked to the microsatellite marker loci *Xgwm212* and *Xgwm292*. Quantitative trait locus (QTL) analysis for cold response revealed that *Vrn-D1* and markers closely linked showed a strong association with the QTL for frost tolerance. This indicated the presence of the predicted locus, *QFr.jic*-5D. However the map position suggests that *QFr.jic*-5D is a linked locus and not a pleiotropic effect of *Vrn-D1*, since the maximum likelihood position was about 6 cM proximal to *Vrn-D1* (Snape et al., 2001b).

The individual mapping data for the 5A and 5D chromosomes showed that the *Vrn-A1* and *Vrn-D1* loci both map at distal locations on homoelogous long arms, and are thus, probably homeoallelic. To prove this further, the maps were aligned using cross hybridising probes and 'bridging' maps of 5A and 5D developed on a double haploid population from the cross of Chinese Spring x SQ1 (Quarrie et al., 1995). This revealed that *Vrn-A1* and *Vrn-D1* are, in all probability, homoeoallelic, as are *Fr1* and *QFr.jic*-5D. These loci are thus probably derived from the common ancestor of the A and D genomes and predate the speciation of bread wheat (Snape et al., 2001b).

In comparison to other *Vrn* genes, RFLP markers linked to *Vrn2* have not been identified, although this gene is important for the adaptation of spring-type wheat in the

southern part of Europe (Iwaki et al., 2002). Furthermore, there have been conflicting reports about their chromosomal location, *Vrn2* on chromosome 2B and *Vrn4* on chromosome 5B (Maystrenko, 1980), *Vrn2* on chromosome 5B (Hoogendoorn 1985b; Iwaki et al., 2001b), and *Vrn4* on chromosome 5D (Kato et al., 1993). Based on the orthologous relationship at *Vrn1*, McIntosh et al. (1998) summarized that the genes formerly designated as *Vrn2* and *Vrn4* are probably the same, or allelic, and that the two genes are designated as *Vrn-B1*. To solve such a confusion, sequence polymorphism at RFLP loci closely linked to *Vrn1* was surveyed by Iwaki et al. (2002), resulting in the establishment of a dCAPS marker WG644-5B linked to *Vrn2* by 1.7 cM. Since *Xwg644* is closely linked to *Vrn-A1* (Galiba et al., 1995; Korzun et al., 1997), it was indicated that *Vrn2* is the ortholog of *Vrn1* on chromosome 5B, i.e. *Vrn-B1*. Although the probe WG644 has not been mapped on chromosome 5B by RFLP analysis, the existence of an orthologous locus was confirmed by the analysis of nulli-tetrasomic lines (Iwaki et al., 2002).

Independence of *Vrn-B1* and *Vrn4* was clearly shown by a test cross between four nearly isogenic lines (NILs) of 'Triple Dirk' (Gotoh, 1979) and by the existence of landraces carrying either *Vrn-B1* or *Vrn4* (Iwaki et al., 2001a). Further analysis is required to clarify the origin and orthologous relationship of *Vrn4*.

1.1.2 Genetic control of photoperiod response

A genetic analysis of flowering control by photoperiod sensitivity has been carried out by many researchers. Both in short day plants, such as rice and maize, and long day plants, such as barley and wheat, genes for photoperiod response determine sensitivity to the length of the photoperiod. In cereals, photoperiod insensitive genotypes enable the initiation of floral primordia without the requirement for long day photoperiods, whereas sensitive genotypes need a long day period for floral primordia initiation (Stracke and Börner, 1998).

In vernalisation sensitive winter wheats, ear emergence time is determined by photoperiod sensitive genes (*Ppd*) and earliness per se (*Eps*) genes that act independently of environmental stimuli. Photoperiod sensitivity is controlled primarily by a homoeologous series of genes *Ppd-D1* (formerly *Ppd1*), *Ppd-B1* (formerly *Ppd2*), and *Ppd-A1* (formerly *Ppd3*) located on chromosome 2D, 2B and 2A, respectively (Welsh et al., 1973; Law et al., 1978).

Scarth and Law (1984) demonstrated that the most potent genes for photoperiod insensitivity are located at the *Ppd-D1* locus on chromosome 2D, followed by *Ppd-B1* locus on chromosome 2B, with the least potent insensitivity alleles being located at the *Ppd-A1*

locus on chromosome 2A. No direct comparisons have been made between the relative strengths of the group 2 photoperiod response loci and those located on other chromosomes.

Comparative studies by Martinic (1975) and Hunt (1979) indicated that winter wheat varieties grown in countries of more northern latitudes including Canada, France and the UK, were usually highly sensitive to photoperiod, whilst those grown in more southern latitudes such as Italia and Yugoslavia, were normally highly insensitive. Inconsistencies were found, however, particularly with older varieties and landraces, where those grown in Southern Europe were much more sensitive than newer introductions.

A similar experiment to know the distribution of genes that influence eco-climatic adaptability whilst determining photoperiodic insensitivity and plant height in European winter wheats was also carried out by Worland et al. (1994). In their experiment, photoperiod sensitivity was measured by the delay in flowering time when plants grown under 9 hours natural light with no supplementary light were compared to plants grown under 9 hours natural lights with 15 hours supplementary low intensity light. Varieties showing delays of less than 50 days were considered day length insensitive, and varieties showing delays of more than 100 days were considered day length sensitive. Under these conditions, they found that all tested Southern European wheats were highly insensitive, and all varieties bred in the UK or Germany were highly sensitive. The division between sensitivity and insensitivity cut across France, with most tested French wheats being insensitive, but with varieties bred for growing to the north of Paris remaining mainly photoperiod sensitive.

Photoperiod insensitivity was first introduced into European wheats by the Italian breeder Nazzareno Strampelli, who used the Japanese variety Akakomugi to reduce the height and bring forward the flowering time of Italian wheats (Strampelli, 1932). Subsequent research showed that the major genes from Akakomugi for height reduction (*Rht8*) and early flowering (*Ppd-D1*) are both located on the short arm of chromosome 2D (Worland and Law, 1985). By 1973 all Italian varieties were of short stature and early flowering Italian varieties were also being grown directly in other south European countries and were being used, along with the Russian varieties Aurora, Bezostaya1, and Kavkas, that had previously obtained photoperiod insensitivity from Italian wheats, to improve the adaptability of locally bred varieties. By 1983 adapted photoperiod insensitive semi-dwarf wheat varieties had spread throughout Southern Europe (Worland et al., 1994).

The primary effect of *Ppd-D1* is to accelerate time to ear emergence. The degree of acceleration is very dependent on environmental conditions. From a standard October sowing in the UK or Yugoslavia, *Ppd-D1* usually accelerates ear emergence time by six to eight days

(Worland et al., 1998). The acceleration is generally less in mid-Germany averaging around two days (Börner et al., 1993).

Analysis of the pleiotropic effects of a *Ppd-D1* allele from the Italian cultivar Mara shows that, beside accelerating ear emergence time, *Ppd-D1* also reduces plant height, tillering and spikelet numbers. Increases in spikelet fertilities more than compensate for reduced spikelet numbers, producing increased numbers of grains per ear (Worland et al., 1998). The ultimate plant yield was determined by the environmental conditions prevailing during the period from grain set to maturity. In Southern Europe, where summers are regularly hot and dry only the early *Ppd-D1* genotype were able to fill their grain prior to the hot dessicating summer conditions. Here, increased numbers of grain per ear combined with larger grains produced regular yield increases of around 30 %. In Mid Europe summers again tend to be hot and dry and *Ppd-D1* promoted average yield increase of around 15 %. In the UK summer conditions are much more variable than in mainland Europe. In traditional cool damp summers the extended grain filling period allows the later flowering *ppd-D1* genotypes, with their grain more evenly distributed over more spikelets, to produce larger grains and higher yields. In the warmer dryer summers of 1989 to 1992, even in UK, the early *Ppd-D1* genotype showed a yield advantage (Worland, 1996).

Scarth, Kirby and Law (1983) have shown that *Ppd-B1* controls ear emergence under short days by regulating the growth of the developing apex, during and after the phase of spikelet initiation. Since there was an opinion that across the middle of Europe including Germany and the UK there may be adaptive advantages for *Ppd-B1* and *Ppd-A1*, single chromosome recombinant lines have been developed by Worland et al. (1998) in a Cappelle-Desprez background between chromosome 2B of Chinese Spring that carries *Ppd-B1* and chromosome 2B of Cappelle-Desprez (*ppd-B1*) and were grown in the UK for harvest in 1993, 1994, 1995 and in Mid-Germany for harvest in 1994 and 1995. The results showed that in England, *Ppd-B1* accelerated flowering time by nearly 5.6 days as compared to 7.6 days for *Ppd-D1* in the same trials, while in Germany *Ppd-B1* accelerated flowering time by 3.5 days compared to 4.2 days for *Ppd-D1* over the same 2 years.

For most recorded characters, the effects of *Ppd-B1* were similar to those of *Ppd-D1* but were slightly reduced with *Ppd-B1*, promoting more modest reductions in height, tillering and spikelet numbers, and smaller increases in grains per spikelet and ear. However, the two years of trialing were not representative of the long term average, with *Ppd-D1* in Germany over the two years reducing yield by an average on 6.3 % compared to an average yield increase of 14.6 % over the previous four years. Therefore more trialing was suggested by

Worland et al. (1998) to determine the true value of *Ppd-B1* to European wheat breeding programmes.

The important role of *Ppd-A1* gene in accelerating heading time was recently reported by Stelmakh (1998). The experiment was carried out by creation of congenic lines in the background of the highly photosensitive winter wheat Mironovskaya 808. The donors of different dominant *Ppd* genes were Bezostaya1 (*Ppd-D1*) and Triple Dirk (two genes nonallelic to *Ppd-D1*, possibly *Ppd-B1* and *Ppd-A1*). The developed lines were tested and characterised in natural and controlled (short and long day) environments under different day lengths (8, 12, 16, 20 and 24 hours). *Ppd-A1* gene always caused maximal earliness and led to the minimal heading delay with shortening photoperiod. Maximal values of these parameters were characteristic for the fully recessive genotype of Mironovskaya 808 and the weakly insensitive *Ppd-B1*, which showed minor differences in earliness.

In another experiment, the set of congenic lines was planted (after vernalising for 60 days) under different photoperiod treatments: growth was started under short (12h) or under long (20h) days and then 20 plants of each genotype were transferred each week to the opposite photoperiod (long or short, respectively) up to heading. When plants were grown initially under long days and transferred to short days, the total number of days to heading decreased progressively with later transference to short days. Range of certain genotype heading times did not change and coincided with the order of their levels of insensitivity, i.e., *Ppd-A1*-*Ppd-D1*-*Ppd-B1*-recessive. Furthermore, it was observed that the carrier of *Ppd-A1* is almost insensitive. Partial sensitivity was noted at early stages for the carriers of *Ppd-B1*, and for the carriers of *Ppd-D1* it became apparent at later stages after 'double ridge' stage (about 30-35 days of initial development).

In wheat, similar genes are frequently located at similar positions on each of the three chromosomes of a homeologous group. The short arm of chromosome 2B is thought to correspond to the long arm of chromosome 2D and the long arm of 2B to the short arm of chromosome 2D (Sears and Sears, 1997). Based on this theory, comparison of the location of *Ppd* gene, *Rht*, *Yr* and *D* gene on both chromosome 2B and 2D was done by Worland and Law (1985). The result showed that if the linkage distances obtained for the genes *Ppd-D1*, *Yr16* and *D4* on chromosome 2D are compared to positions of *Ppd-B1*, *Yr5/Yr7* and *D2* on chromosome 2B they fall in very similar positions suggesting that the genes *Ppd-D1* and *Ppd-B1*, *Yr16* and *Yr5/7*, and *D4* and *D2* are homoeoallelic.

At this moment the exact location of *Ppd-D1* and *Ppd-B1* on chromosome 2D and 2B, respectively have been known using the power of molecular marker combined with a precise

genetic stock. Using 100 single chromosome recombinant lines between chromosome 2B homologues of Cappelle-Desprez and Chinese Spring in a Cappelle-Desprez background, Worland and Sayers (1996) established a genetical linkage map for these 2B recombinant lines showing the location of *Ppd-B1* in relation to 7 RFLP markers. A linkage map of chromosome 2D constructed earlier using single chromosome recombinant lines of the varieties ´Cappelle' and ´Mara' put *Ppd-D1* 20.9 cM proximal to the gene *Rht8* (Worland et al., 1998a). To date, many microsatellites are available on each chromosome of bread wheat (Röder et al., 1998b; Petsova et al., 2000). By combining data found previously with new microsatellite markers, a complete molecular linkage map of chromosome 2D of wheat was established recently by Petsova and Röder (2002).

It was mentioned that genes on other wheat chromosomes including chromosome 6B have been implicated in determining photoperiod response (Worland, 1996). Substitution line analysis has not revealed photoperiod response effects on other homoelogous groups in wheat (Snape et al., 2001a). However, comparative mapping in barley by Laurie et al. (1995) shows that genes for photoperiod response, *Ppd-H1* and *Ppd-H2* exist on chromosome 2H and 7H, respectively, and Stracke and Börner (1998) have mapped a photoperiod locus on barley 6H. Cross hybridising markers show that *Ppd-H1* on 2H is homologous to the wheat *Ppd*-1 series of genes. Thus, extending this analysis further would predict that a *Ppd*-2 and *Ppd*-3 series should exist on wheat group 6 and 7 chromosomes. However, no analysis using conventional crosses or chromosome substitution lines has yet revealed allelic variation for photoperiod response on these chromosomes, and they deserve further attention in this respect (Snape et al., 2001a).

1.1.3 Genetic control of developmental rate

The presence of earliness per se (*Eps*) genes that influence flowering time independently of environmental stimuli were clearly demonstrated by Martinic (1975) who found that due to *Eps* genes some photoperiod sensitive varieties flowered before photoperiod insensitive varieties. Similarly Hoogendoorn (1984) estimated that in the UK earliness per se effects on ear emergence time were as great as photoperiod differences.

The *Eps* genes are widely distributed amongst European wheat and are recognised by alterations in ear emergence time of vernalised plants grown under long days where all environmental stimuli requirements should have been satisfied. Under this condition the photoperiod insensitive varieties flowered a significant 10.16 days earlier than photoperiod

sensitive varieties. The earlier flowering was correlated with a mean reduction of 2.68 spikelets (Worland et al., 1994).

In comparison to the photoperiod and vernalisation response genes, the genetic map locations and physiological effects of *Eps* genes are poorly defined. However, the physiological basis is probably through alteration of numbers of vegetative and floral primordia being initiated or the rate of initiation (Worland et al., 1994). Genes influencing this character have been located on chromosome 2BL (Scarth and Law, 1983), 7B (Flood and Halloran, 1983) and 3A, 4B, 4D and 6B (Hoogendoorn, 1985a).

At present only the *Eps* genes located on group 2 chromosomes are being studied in detail. The effects of other genes particularly those widely reported on chromosome 3A need investigating (Worland, 1996). Using single chromosome recombinant lines developed between chromosome 2B homologues from Marquis and Chinese Spring in a Chinese Spring background, Scarth and Law (1983) were able to detect *Ppd-B1* segregating on the short arm and a second gene that influenced flowering time independently of the environment on the long arm associated with disease resistance genes *Yr7*, *Sr9g* and *Sr16*. Whilst Chinese Spring carried the early allele for *Ppd-B1*, Marquis carried the early allele for earliness per se.

A second factor influencing flowering time was detected together with *Ppd-D1* gene on the same chromosome by Worland and Law (1985) using single chromosome recombinant lines between 2D homologues from Cappelle-Desprez and Mara in a Cappelle-Desprez background. This second factor was associated with *Yr16*, a gene for adult plant resistance to yellow rust. Whilst *Ppd-D1* accelerated flowering time by around eight days with pleiotropic effects on reducing height and spikelet numbers the long arm factor accelerated flowering time by about one day, reduced spikelet numbers by 0.4, promoted increases in spikelet fertilities and in environments like Yugoslavia significant increases in yield.

The *Eps* genes tend to have relatively small effects and to date have been mapped only as QTL. This is partly because most have been identified in crosses made to study the segregation of major vernalisation or photoperiod response genes. Because they have been located as QTL, it is not possible to undertake a comparative analysis of *Eps* effects with any confidence (Laurie, 1997). However, the effect of *Eps2* gene in Barley (Laurie et al., 1995) is paralleled in wheat cross (Worland, 1996) suggesting the presence of homologous genes. Furthermore Snape et al. (2001a) predicted that group 3, 4, 5, 6 and 7 chromosomes of wheat also carry *Eps* genes based on comparative analysis with barley. Therefore, Laurie (1997) suggested that in order to identify possible homologies easier, the exact map location of the wheat and barley genes should be defined. Undoubtedly, it will be possible to ‘fine time’

flowering time for regional variations in climate using such loci once their primary and pleiotropic effects have been characterised (Snape et al., 2001a).

1.2 Marker-assisted selection

Marker-assisted selection (MAS) is based on the concept that it is possible to infer the presence of a gene from the presence of a marker that is tightly linked to the gene. If the marker and the gene are located far apart then the possibility that they will be transmitted together to the progeny individuals will be reduced due to the double cross over recombination events (Kumar, 1999). Hence, the essential requirements for MAS in a plant breeding program are (Gupta et al., 1998):

1. Marker(s) should co-segregate or be closely linked (1 cM or less) with the desired trait.
2. An efficient means of screening large populations for the molecular markers should be available.
3. The screening technique should have high reproducibility across laboratories, be economical to use and should be user friendly.

Once a tight linkage is found between a molecular marker and a gene of interest, the inheritance of the gene can be traced in breeding programs. Therefore, the identification of markers linked to important traits could result in the development of more efficient breeding strategies, enabling breeders to discard unwanted genotypes early in the program.

Recently, Koebner and Summers (2003) summarize the three main advantages of marker-assisted selection (MAS) i.e.:

1. It becomes possible to select, on a single plant basis, for a trait (or combination of traits) in situation in which this is conventionally ineffective either because environmental variation or because phenotypic–based assessment is difficult or not cost-effective.
2. For traits that are under multigenic control, the individual genetic components can be maintained and ultimately fixed in the homozygous state at the end of the breeding process.
3. Both recessive genes, and those not readily amenable to phenotypic selection, can be maintained in segregating generations without the need for validation at each generation via a progeny test.

The choice of marker system to be used for the detection of DNA polymorphism, depends on the objective of study, the need dictated by its specific application and on the

facilities and skill available in a laboratory (Gupta et al., 1999; Mohler and Wenzel, 2003). Powell et al. (1996) added that cost, convenience and technical feasibility should also be considered before making this decision. They gave examples that while SSRs and AFLPs, despite the high cost of their development, are popular for their efficiency, the RAPDs are popular because of their simplicity and low costs, even if they are the least efficient.

Molecular markers are virtually unlimited in their number, detecable at all plant developmental stages showing no pliotropic effects. However, one of their biggest advantage is they can be used to dissect quantitatively inherited traits into single Mendelian factors. Thus, molecular markers are of great value in applying genomics to crop improvement (Tanksley, 1993; Mohan et al., 1997; Wenzel, 1998; Snowdon et al., 2002).

In order to maximise its potential, MAS requires assays that are fast, reliable and easy to implement. PCR-based markers, such as sequence tagged site (STS) (Olson et al., 1989) markers are particularly suited to MAS. The conversion of linked markers to STS overcomes problems such as time-consuming protocol associated with RFLPs and AFLPs, and the lack of reliability with RAPD markers. There are several examples where RAPD, RFLP and AFLP markers have been converted to STS markers for use in MAS (Paran and Michelmore, 1993; Schachermayr et al., 1995; Feuillet et al., 1995; Dedryver et al., 1996; Talbert et al., 1996; Blair and McCouch, 1997; Huang et al., 1997; Paltridge et al., 1998; Toojinda et al., 1998; Mohler et al., 2001).

Before using the molecular markers in actual plant breedings, it is necessary to undertake studies on marker validation, a process of examining the behaviour of markers and the associated polymorphism in different genetic backgrounds (Langridge and Chalmers, 1998). If closely flanking markers can be applied in other crosses or even in related species (Mohler et al., 2002), they will greatly increase the efficiency of marker assisted selection. For example, several genomic regions associated with plant height, ear height, anthesis and silk emergence in maize have been identified in more than one population as well as in populations of sorghum (Austin and Lee, 1996).

1.2.1 Isozymes and morphological markers

Molecular diversity has been studied in plants for about three decades. The development of genetic linkage maps originated with the use of naturally occurring variation between enzymes and morphological traits, which could be used to detect and map single trait loci in segregating populations (Tanksley et al., 1982). Enzyme polymorphisms have been intensively studied in many species since the 1960s. Through multidisciplinary investigations

of different enzyme encoding genes in *Drosophila melanogaster*, *Fundulus heteroclitus* and several other species revealed that enzyme allelic variants are differentially affected by natural selection (Powers et al., 1991).

Isozyme markers are generally co-dominant and provide a useful source of markers for the development of linkage maps prior to the establishment of alternative molecular marker approaches. Once map positions of isozyme genes had been established, they could be used to locate other genes which may be linked (Tanksley and Rick, 1980). However, their use is limited due to their limited number in any crop species and also because they are subject to post translational modifications (Staub et al., 1982).

Similarly, morphological markers were used to generate early linkage maps, but their usefulness is limited by the low number of morphological markers which are available to the plant breeder for crop improvement programmes. In addition, the expression of morphological markers is affected by environmental conditions (Mohan et al., 1997). Beside environment, the expression of such markers is also altered by epistatic and pleiotropic interactions (Kumar, 1999). Overall, isozyme and morphological markers only offered limited genome coverage, were time consuming to assay, and detected relatively low levels polymorphisms compared with more recently developed marker systems.

1.2.2 Restriction Fragment Length Polymorphism

The development of restriction fragment length polymorphism (RFLP) markers revolutionised plant genome mapping, offering a new source of virtually unlimited co-dominant markers with extensive genome coverage (Beckmann and Soller, 1983). Among the various molecular markers developed, RFLPs were developed first and initially used for human genome mapping (Botstein et al., 1980) and later they were adopted for plant genome mapping (Weber and Helentjaris, 1989). The approach involves digesting DNA with restriction enzymes, separating the resultant DNA fragments by gel electrophoresis, blotting the fragments to a filter and hybridising probes to the separated fragments (Castagna et al., 1994).

Probes are obtained by either synthesising specific regions of the genome using cloned DNA, or by testing clones from a random DNA library and selecting those which are single or low copy. If two individuals differ for a particular restriction site that affects the size of the DNA fragment homologous to the probe, then a band will appear at different location in their respective autoradiographs. Variation in restriction sites can therefore be detected as restriction fragment length polymorphisms. Similarly, insertions or deletions between

restriction sites will also generate fragment length polymorphisms (Beckman and Soller, 1986).

As a technique for diversity studies, there are three important advantages which should be considered. The first is that RFLPs are highly reproducible between laboratories and the diversity profiles generated can be reliably transferred. The second is that RFLPs are co-dominant markers, enabling heterozygous to be distinguished from homozygous. The third advantage is that no sequence-specific information is required and, provided suitable probes are available, the approach can be applied immediately for diversity screening in any system (Karp and Edwards, 1997).

RFLP analysis has been used extensively in the construction of genetic maps and has been successfully applied to genetic diversity assessments, particularly in cultivated plants such as barley (Graner et al., 1991), einkorn wheats (Castagna et al., 1994), sorghum (Deu et al., 1994) and oil palm (Jack et al., 1995), but also in populations and wild accessions such as rubber plant (Besse et al., 1994), cocoa (Laurent et al., 1994), and bulb onion (Bark and Harvey, 1995). Other applications of RFLP markers include varietal identification and the introgression of alleles from wild or related species into breeding programs. Although RFLPs have played an important role in developing linkage maps, they are not ideally suited to a large scale diagnostic applications (Rafalski and Tingey, 1993). This is primarily because they are labour intensive, time consuming, and require a relatively large amount of DNA to perform the assay. Karp and Edwards (1997) added that this technique is also not suitable for some plant systems, where extraction is problematic because of the presence of polyphenols or polysaccharides which complex with the DNA, or where only very limited amounts of source material are available.

1.2.3 Random Amplified Polymorphic DNA

This PCR-based technique uses short primers of arbitrary sequence to produce random amplification of DNA fragments from the genome being studied (Williams et al., 1990). Polymorphisms detected among the amplified products arise as a result of changes in specific nucleotide sequence information in one or both of the priming sites. These changes are visible as the presence or absence of a particular RAPD band, or variation in size of the amplified fragment when the products are separated by gel electrophoresis (Rafalski and Tingey, 1993).

RAPDs have been used for a variety of purposes including the construction of genetic linkage maps (Reiter et al., 1992), gene tagging, identification of cultivars (Nybom, 1994),

assessment of genetic variation in populations (Chalmers et al., 1992) and species (Nesbitt et al., 1995; Fu et al., 2002), the development of species-specific (Chen et al., 1998), genome-specific and chromosome-specific markers (Wang et al., 1995) and more importantly for identification and selection of the desired genotype (for a variety of traits of economic importance) in segregating populations during breeding programmes, as example characterization of CMS and maintainer lines (Ichii et al., 2003).

The enormous attraction of RAPDs is that there is no requirement for DNA probes or for any sequence information for the design of specific primers. The procedure involves no blotting or hybridising steps. The technique is, therefore, quick, simple, and efficient and only requires the purchase of thermocycling machine and agarose gel apparatus to set up in a laboratory for any new system under study. It requires small amounts of DNA (10 ng per reaction) and sample throughput can be quite high. The procedure can also be made automatic with extremely high throughput. RAPDs have also been proved to detect higher levels of polymorphism compared with RFLPs in case where the two techniques have been applied to the same material (Hadrys et al., 1992).

When they were initially developed, it was thought that RAPDs had the potential to provide a new source of unlimited genetic markers for use in the construction of genetic maps. However studies have shown that RAPDs have several drawbacks, including their dominant mode of inheritance (Rafalski and Tingey, 1993) as well as problems associated with their reproducibility (Talbert et al., 1994; Van Eck et al., 1995; Nagaoka and Ogihara, 1997). Other limitations relate to the extent to which products can be considered to be allelic when derived from different varieties and species. Furthermore, it has been noted that the short arbitrary primers may amplify sequences homologous to repetitive DNA in complex genomes (Powell et al., 1995). However, once a marker is found linked to a trait of interest, it is possible to convert the RAPD assay into a more reproducible PCR-type assay using techniques such as allele specific PCR or sequence characterized amplified region (SCAR) assays (Rafalski and Tingey, 1993). This approach has been used successfully in wheat (Dedryver et al., 1996), lettuce (Paran and Michelmore, 1993), and lentil (Chowdhurry et al., 2001).

1.2.4 Amplified Fragment Length Polymorphism (AFLP)

The development of amplified fragment length polymorphisms (AFLPs) produced another source of PCR based markers with the potential to rapidly saturate genetic maps. The technique is based on selective PCR amplification of restriction fragments generated by

specific restriction enzymes and oligonucleotide adapters of few nucleotide bases. The PCR-primers consist of a core sequence (part of adapter), a restriction enzyme specific sequence and a number of selective nucleotides. Usually three selective nucleotides for each of the two primers are used for regular plant genomes, but for species with small or large genomes this number can be adjusted accordingly. The AFLP technique simultaneously generates fragments from many genomic sites (typically 50-100 fragments per reaction) that are separated by gel electrophoresis and generally yield highly informative fingerprinting profiles (Vos et al., 1995).

AFLP is similar to RFLP except that it uses PCR amplification instead of southern hybridisation for the detection of restriction fragments requiring much less template DNA. AFLPs also have much higher multiplex ratio than RFLPs. DNA of any origin or complexity can be used with the number of restriction fragments detected in complex genome being virtually unlimited. In contrast to RAPDs, almost every AFLP reaction is useful, with large numbers of polymorphic bands common (Mackill et al., 1996). Additionally, the majority of AFLP fragments correspond to unique positions on the genome, and can be used as markers in genetic and physical maps. AFLPs have the capacity to identify large numbers of loci, increasing their ability to detect polymorphism compared to other PCR-based methods such as RAPDs and SSRs (Thomas et al., 1995).

The distribution of AFLP markers within the plant genomes differs according to enzyme combinations used in the assays. Methylation insensitive enzymes, including *Eco*RI and *Mse*I generate fragments that tend to cluster in centromere regions, whereas fragments generated using methylation sensitive enzyme combinations, including *Pst*I and *Msp*I are distributed more evenly throughout genomes (Castiglioni et al., 1999; Young et al., 1999). Since expressed plant genes are generally hypomethylated (Martienssen and Richards, 1995), using methylation sensitive enzymes in AFLP analysis may also increase the possibility of identifying markers that are tightly linked to target genes.

The AFLP process may be improved by using a multifluorophore detection technique (Schwarz et al., 2000). In comparison to conventional ^{33}P-based AFLP analysis the multifluorophore technique allows multimixing as well as simultaneous amplification of AFLP fragments with different primer combinations in one reaction (multiplexing).

The high efficiency, reproducibility and reliability of AFLP have been supported by a number of recent publications, such as for the analysis of genetic linkage and gene mapping (Mackill et al., 1996; Vorrips et al., 1997; Hartl et al., 1999; Zeller et al., 2002), map based cloning (Cnops et al., 1996; Schwarz et al., 1999), plant evolution (Heun et al., 1997),

biodiversity studies (Barret & Kidwell, 1998; Zhu et al., 1998), and aligning genetic maps from different genotypes (Waugh et al., 1997; Singrün et al., 2003). Abundant AFLPs have been found in many plant species (Shan et al., 1999; Gupta et al., 1999) confirming their use in plant genetic studies.

Although the AFLP technique is powerful and reliable in identifying markers closely linked to genes of interest, it has some disadvantages for use in MAS and map-based cloning. Limitations to the large scale, locus-specific application of AFLPs include their dominant type of inheritance, the intensity of labour involved, and the high costs. Hence, conversion of AFLP markers into sequence specific polymerase chain reaction (PCR) markers is required for screening large breeding populations at low costs (Dussle et al., 2002).

1.2.5 Microsatellites

Microsatellites or simple sequence repeats (SSRs) are tandemly repeated motifs of 1-6 bases found in all prokaryotic and eukaryotic genomes analysed to date. They are present in both coding and noncoding regions and are usually characterized by a high degree of length polymorphism (Zane et al., 2002). Their abundance, codominant nature and high level of variability make them a suitable assay for detecting variation in phylogeny and population studies (Lagercrantz et al., 1993; Schug et al., 1997; Harr et al., 1998)

The flanking sequences of SSRs are often unique, allowing primers to be designed that result in tagged SSR markers representing a single locus. The majority of the allelic variation of SSRs is thought to arise as a result of slip strand mis-pairing (SSM). SSM involves denaturing and displacement of strands of DNA duplex followed by mis-pairing of complementary bases at the site of an existing tandem repeat. When followed by replication or repair, this can lead to insertions or deletions of one or several of the short repeat units (Levinson and Gutman, 1987). Unequal crossing over can also generate tandem duplications in DNA, as well as insertion or deletion events in the sequence that flank the SSR region (Grimaldi and Crouau-Ray, 1997). All of these mechanisms can potentially generate allelic variation, providing an important source of highly polymorphic markers. This variation is detected by PCR amplification using primers complimentary to the flanking sequences. Unlike RAPDs, SSRs as a marker system have been shown to be highly reproducible between laboratories (Jones et al., 1997).

The number and composition of microsatellite repeats differ in plants and animals. The frequency of repeats longer than 20 bp, has been estimated to occur every 33 kb in plants unlike mammals where it is found to occur every 6 kb (Wang et al., 1994). In humans, AC or

TC is a very common repeat unit, but in plants AT is more common followed by AG or TC (Powell et al., 1996) but in general plants have about 10 times less SSRs than humans.

The first report of microsatellite in plants was made by Condit and Hubbel (1991), suggesting their abundance in plant systems. Later, Akkaya et al. (1992) reported length polymorphisms of SSRs in soybean, which opened a new source of PCR-based molecular markes for other plant genomes. At this moment, it has been demonstrated that microsatellites are highly informative and locus-specific markers in many species (Song et al., 2002; Röder et al., 1998b).

In wheat, it has been shown that microsatellites show a much higher level of polymorphism and informativeness than any other marker system (Plaschke et al., 1995; Röder et al., 1995; Ma et al., 1996; Bryan et al., 1997). In recent years, efforts have been made to develop markers based on (AG/TC)n and (AC/TG)n repeats in wheat and about 315 of these markers are now available (Korzun et al., 1997; Röder et al., 1998b; Petsova et al., 2000a, b, c). The number of microsatellites markers are predicted to increase soon following the finding of trinucleotide microsatellites characterized by Song et al. (2002). They concluded that (TAA/ATT)n microsatellites would provide the most abundant and the most polymorphic source of trinucleotide microsatellite markers in wheat.

Although microsatellites are always reported to be highly informative and reproducible, co-dominant inheritance and locus specificity (Powell et al., 1996), they have also several drawbacks, including the high cost and length of time required for their development. These are caused by the need to be isolated de novo from most species being examined for the first time (Zane et al., 2002). However, although the initial cost may be significant, once developed the cost of implementing these markers is greatly reduced. In addition, they are easily transferable between laboratories as the sequence information can be distributed, allowing other research groups to generate their own primers.

1.2.6 Single Nucleotide Polymorphism

The primary candidate for the next generation of marker is the single nucleotide polymorphism (SNP). At it simplest, an SNP consists of a single base difference within a given segment of DNA between two individuals (Koebner and Summers, 2003). SNPs and insertion-deletions, which are the basis of most differences between alleles, are an essentially inexhaustible source of polymorphic markers for use in the high resolution genetic mapping of traits, and for association studies that are based on candidate genes or possibly whole genomes (Rafalski, 2002a).

One estimates that comparing two human DNA sequences results in a SNP every 1000-2000 nucleotides. That may not sound like much until we realize that there are 3.2 billion nucleotides in the human genome, which translates into 1.6 million-3.2 million SNPs (Stoneking, 2001). In maize, even conservative estimates would predict over 20 million polymorphisms to be available for analysis (Rafalski, 2002 b).

Koebner and Summers (2003) mentioned that the overwhelming attractions of SNPs can be divided into two fold: (1) they offer the potential for a high density of markers. The relevance of this to MAS is that it should be possible to find an informative marker in the right region in any segregating situation, even if the probability of finding polymorphism at any one SNP locus is low; and (2) the SNP output is of the binary type and this presents an easier target for automated data interpretation than the length-based outputs that are typical of STMS. In addition such data need not be generated by electrophoresis, giving the potential for simpler and cheaper analytical platforms.

A dominant SNP detection technology has not emerged so far. The technologies are clearly in flux, with many competing approaches being available. However as the prices of DNA sequencing and SNP assays drop, the argument for SNPs will become more compelling. A recent report states that in the past year, the average cost of a SNP genotype has been reduced from approximately US$ 1.00 to US$ 0.10, but that a further order of magnitude reduction to US$ 0.01 per assay will be required before wide-scale usage of the technology becomes feasible (Roses, 2002).

Direct sequencing of DNA segments from several individuals is the most direct way to identify SNP polymorphisms (Gaut and Clegg, 1993). PCR primer is designed to amplify 400-700 bp segments of DNA, which are frequently derived from genes of interest or ESTs (Rafalski, 2002a). A rapid and simple method to reliably identify and score SNPs in DNA samples was recently developed by employing DHPLC technology (Oefner and Underhill, 1998; Bäümler et al., 2003a). Using this technique, Schwarz et al. (2003) reported that the cost effective and high throughput genotyping DHPLC technique is particularly suitable for routine diagnosis of SNPs in homologous HMW glutenin gene sequences. This technique still showed it's superior, even if compared with CAPS marker analysis. The automated sample feeding allows analysis of up to 200 isolates per day (Bäumler et al., 2003b).

2 Materials and methods

2.1 Plant material

Plant material used in this research consists of four populations segregating for three adaptation genes i.e. *Ppd-A1*(*Ppd3*), *Ppd-B1*(*Ppd2*) and *Vrn-D1*(*Vrn3*). Phenotypic classifications of these populations were made in the green house by A.J.Worland (John Innes Centre (JIC)) and in the field by Janny van Beem (International Maize and Wheat Improvement Centre (CIMMYT)). Both of them classified the lines of the populations into two categories: early and late in flowering. An additional category, intermediate, was also made by CIMMYT to show that certain lines have characters in between early and late in flowering. The information of plant status and numbers of lines of each population are shown in Table 3.

Table 3. Information of plant status used in this research

Gene	Chromosomal Location	Population	Population Type	No of Individuals
Ppd-B1 (*Ppd2*)	2B	Mercia x Mercia(CS2B)	SCDH[2]	72
Ppd-B1(*Ppd2*)	2B	CS[1] x CS(Marquis2B)	SCRL[2]	70
Ppd-A1(*Ppd3*)	2A	Mercia x Mercia(C5912A)	SCDH[2]	74
Vrn-D1(Vrn3)	5D	CS x CS(Hope5D)	SCRL[3]	94

CS[1]= Chinese Spring

SCDH[2]= single chromosome double haploids

SCRL[3]=single chromosome recombinant lines

For assembling of phenotypic pools, only plants which have been strictly classified as early and late in flowering, respectively, were chosen from the segregating populations. Two bulks were made by mixing equal amounts of genomic DNA each from the same number of early flowering lines as well as late flowering segregants.

Molecular marker validation was carried out with the DNA from 36 lines which have been confirmed (by test crosses made at CIMMYT) for the presence of the respective genes. A useful marker showed high correlation between the presence of a specific marker allele and the respective known gene configuration.

All of the markers found were localized in the wheat genome using nulli-tetrasomic (NT) lines of cultivar Chinese Spring (CS) (Sears, 1954). Wheat chromosome-specific markers were identified as bands missing in only one NT stock but present in all other NTs

and in CS. Furthermore, all polymorphisms retrieved from early flowering segregants were subjected to analysis of a set of nine chromosome deletion lines for the short arm of chromosome 2B (Table 4), which was kindly provided by Dr John Raupp (Department of Plant Pathology, Kansas State University). All of the aneuploid lines were developed in the genetic background of CS wheat. These lines are maintained at the Wheat Genetics Resource Center (WGRC), Department of Plant Pathology, Kansas State University, Manhattan, Kansas, United States of America.

Table 4. Description of deletion lines for the short arm of chromosome 2B

Deletion lines	Fraction length	Description
2BS-7	0.89	20″ + 1″[del2BS-7]
2BS-14	0.83	19″ + 1″[del2BS-14] + 1″[del1AL-6]
2BS-5	0.79	18″ + 1″[del2BS-5] + 1″[del6BS-1] + t″[6BS]
2BS-3	0.75	20″ + 1″[del2BS-3]
2BS-6	0.56	19″ + 1″[del2BS-6] + 1′[del7AS-9] + 1′[7A]
2BS-1	0.53	20″ + 1″[del2BS-1]
2BS-9	0.40	19″ + 1″[del2BS-9] + 1″[del7AS-11]
2BS-11	0.27	20″ + 1″[del2BS-11]
2BS-2	0.15	19″ + 1″[del2BS-2]

The fraction length (FL) value in a given deletion line identifies the breakpoint in the deleted chromosome and the length of the remaining chromosome arm from the centromere relative to the length of the complete arm. Calculation of FL value was described in detail by Endo and Gill (1996).

The chromosome bin assignment in this study indicates the physical location of each deletion intervals in a chromosome according to the FL values. As examples, the deletion lines 2BS-7, 2BS-14, 2BS-5 are missing 11 %, 17 % and 21 % of terminal segments of the 2BS arm, respectively. Since 9 deletion lines (2BS-7, 2BS-14, 2BS-5, 2BS-3, 2BS-6, 2BS-1, 2BS-9, 2BS-11, 2BS-2) were available for the experiments, the assignment of the markers was also done among these intervals. Therefore, these 9 breakpoints divide the short arm of chromosome 2B into ten chromosome bins.

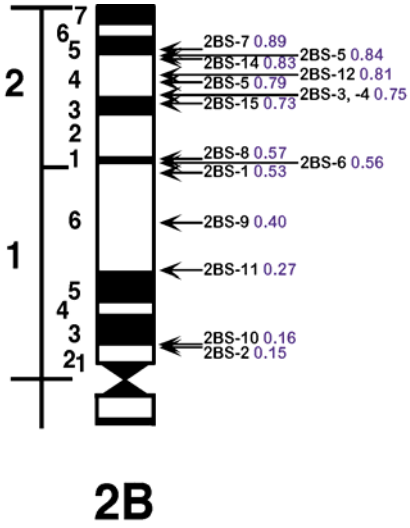

Figure 1. The physical maps of the short arm of chromosome 2B of wheat. Fraction-length values and deletion lines are indicated on the right.

2.2 Enzymes and solutions

2.2.1 Enzymes

Type II restriction endonucleases

Name	Restriction site	Supplier
*Eco*RI	5′-G↓AATTC-3′	New England Biolabs
*Mse*I	5′-T↓TAA-3′	New England Biolabs
*Sse*8387I	5′-CCTGCA↓GG-3′	Amersham
*Hin*6I	5′-G↓CGC-3′	Fermentas
*Avi*II	5′-TGC↓GCA-3′	Fermentas

Remaining enzymes

Name	Supplier
T4 DNA ligase	Biolabs
Taq DNA polymerase	New England Biolabs
10 mg/ml RNase A	Qiagen

2.2.2 Basic solutions

- 40 mg/ml Ampicillin (in H_2O)
- 10 % Ammoniumpersulfate (APS)

- Loading buffer for agarose gel electrophoresis
  0.2 M EDTA
  40 % (v/v) glycerine
  0.03 % (w/v) bromophenolblue
- Loading buffer for polyacrylamid gel electrophoresis

98 %	Formamide	Amresco
0.005 %	Dextran Blue	Fluka

- Chloroform/isoamyl alcohol (24:1)
- 0.5 M EDTA pH 8.0
- 10 mg/ml Ethidium bromide
- 5 M NaCl
- 3 M Ammonium acetate (NH_4Ac)
- 1 M Tris pH 7.5
- 1X TAE buffer For 1000 ml:
  40 mM Tris 4.84 g
  20 mM Hac 1.142 ml cold hydroxy acetate
  5 mM EDTA 0.372 g EDTA
- 1X TBE buffer For 1000 ml:
  89 mM Trisbase 10.8 g Trisbase
  89 mM boric acid 5.5 g boric acid
  2 mM EDTA 0.83 g EDTA
- 1X DNA storage buffer For 1000 ml:
  10 mM Tris-Cl pH 8.0 10 ml 1 M Tris (pH 8.0)
  1 mM EDTA pH 8.0 2 ml 0.5 M EDTA (pH 8.0)
  988 ml H_2O

2.2.3 Solutions for DNA isolation

- 1.5X CTAB For 1000 ml:
  1.5 % (w/v) CTAB 15 g CTAB
  150 mM Tris-Cl 150 ml 1 M Tris-Cl pH 7.5
  1.5 mM EDTA 30 ml 0.5 M EDTA (pH 7.5)
  105 mM NaCl 210 ml 5 M NaCl
- 1.5 % β-mercaptoethanol

- 75 % EtOH

2.2.4 Solutions for AFLP analysis

- 1X $TE_{0.1}$ buffer
  20 mM Tris
  0.1 mM EDTA

2.3 DNA isolation

Total genomic DNA was isolated according to the protocol of Saghai-Maroof et al. (1984) with minor modifications. Briefly, 3-5 g of leaf tissue per sample were ground in liquid nitrogen and incubated at 60°C for 30 min with 15 ml of 1.5X CTAB (cetyl-trimethylammonium bromide) extraction buffer in 50–ml polypropylene tubes. After 5 min cooling on ice, 15 ml 24:1 chloroform:isoamyl alcohol was added. Samples were incubated for 30 min by shaking and then centrifuged at 2100 x g for 30 min. The aqueous layer was transferred to a new tube and 20 µl RNase A (10 mg/ml) was added. Samples were incubated for 30 min at room temperature. About one volume of cold isopropanol was added to precipitate DNA. After 30 min incubation at 4°C, precipitated DNA was withdrawn with a glass rod and placed in a 2-ml reaction tube containing 1 ml of 75 % ethanol. After washing twice with 75 % ethanol, the second time overnight, the washing solution was removed and the DNA pellet was dried thoroughly and dissolved in TE buffer. DNA samples were stored at –20°C. The DNA was diluted to a concentration of 100 ng/ µl and 50 ng/µl before used in AFLP and SSR experiments, respectively.

2.4 PCR-based molecular marker techniques

2.4.1 AFLP analysis

The AFLP protocol was carried out as described by Vos et al. (1995) with modifications according to Schwarz et al. (2000).

2.4.1.1 Restriction-Ligation

0.5 µg of genomic DNA was digested either with one unit *Mse*I and five units *Eco*RI or one unit *Mse*I and five units *Sse*8387I. Five pmol *Eco*RI adaptor and *Sse*8387I adaptor, respectively and 50 pmol *Mse*I adaptor were ligated with 1 U T4 DNA ligase (all enzymes New England Biolabs) in a buffer containing 10 mM Tris-HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 1mM ATP and 50 ng/l bovine serum albumine in a total volume of 11 µl for 3h at 37°C.

The DNA samples were diluted with $TE_{0.1}$ buffer to a final volume of 200 µl and stored at –20°C. The sequence of *Eco*RI adaptor was 5′-CTCGTAGACTGCGTACC-3′, 3′-CTGACGCATGGTTAA-5′and the sequence of the *Mse*I adaptor was 5′-GACGATGAGTCCTGAG-3′, 3′-TACTCAGGACTCAT-5′. The sequence of *Sse*8387I adaptor was 5′-CTCGTAGACTGCGTACATGCA-3′, 3′-CATCTGACGCATGT-5′.

2.4.1.2 Preselective amplification

Preselective amplification of target sequences was performed either with *Eco*RI and *Mse*I adaptor-homologous primers, each possessing one additional nucleotide at the 3′ end, or *Sse*8387I and *Mse*I adaptor-homologous primers that did not have any selective nucleotide at the 3′ end. Polymerase chain reactions were set up with 4 µl diluted restriction-ligation DNA, 2.5 pmol *Eco*RI +A primer and 2.5 pmol *Sse*8387I + 0 primer, respectively, 2.5 pmol *Mse*I + C Primer and 2.5 pmol *Mse*I + 0 primer, respectively, 0.4 U *Taq* DNA polymerase (Qiagen GmbH), 0.2 mM of each dNTP (Amersham- Pharmacia Biotech) and 1x Qiagen PCR buffer in a volume of 20 µl. The PCR reaction was performed in a PE 9600 thermal cycler (Perkin Elmer) programmed for 20 cycles at 94°C (1 s), 56°C (30 s), 72°C (2 min). To verify successful amplification, 10 µl of the PCR mixture was electrophoresed on a 1.5% agarose gel in 1x TAE buffer stained with 0.5 µg/ ml ethidium bromide: a smear of amplified target fragments was visible in the range 100–1500 bp. The remaining 10 µl were diluted 20-fold by adding 190 µl TE 0.1 buffer, and stored at –20°C.

2.4.1.3 Selective amplification

Selective amplification was achieved with two types of primer combinations i.e. *Eco*RI +ANN and *Mse*I+CNN and *Sse*8387I +NN and *Mse*I +NN. Only *Eco*RI and *Sse*8387I primers were labeled using either 5-carboxy-fluorescein (5-FAM), 2′,7′-dimethoxy-4′,5′-dichloro-6 carboxy-fluorescein (JOE), or N,N,N′,N′-tetramethyl-6-carboxy-rhodamin (TAMRA). Polymerase chain reactions were carried out using 3 µl diluted pre-amplified DNA, 1 pmol labeled *Eco*RI + ANN primer and *Sse*8387I+NN, respectively, 5 pmol unlabeled *Mse*I + CNN primer and *Mse*I+NN, respectively, 0.4 U *Taq* DNA polymerase, 0.2 mM of each dNTP and 1 x Qiagen PCR buffer in a total volume of 20 µl.

For amplification, the following cycle profile was used: one cycle of 30 s at 94°C, 30 s at 65°C, 2 min at 72°C, followed by 8 cycles in which annealing temperature was subsequently lowered 1°C per cycle, and finally 23 cycles of 1 s at 94°C, 30 s at 56°C, 2 min at 72°C. For sample loading, 2 µl PCR products were mixed with 0.15 µl of 6-carboxy-X-rhodamin (ROX)-labeled internal length standard GeneScan-500 ROX (Applied Biosystems) and 0.85 µl formamide dye, denatured for 3 min at 90°C and chilled on ice.

Electrophoresis of 36 or 48 samples was carried out using 5% denaturing polyacrylamide gels (Long Ranger^{TM}, FMC Bioproducts) in 1x TBE electrophoresis buffer on an ABI Prism^{TM} 377 DNA sequencer (Applied Biosystems) at 2500 V for 4h. For raw data collection, the ABI PRISM^{TM} V.1.1 collection software was used. AFLP fragments were analysed using GENESCAN^{TM} analysis software version 2.1 (Applied Biosystems) as described in the user's manuals.

Survey of phenotypic pools for AFLP polymorphism was carried out with 256 *Eco*RI+ANN-*Mse*I+CNN primer combinations and 75 *Sse*8387I+NN-*Mse*I+NN primer combinations. The polymorphisms found were then used to score the mapping populations.

2.4.2 Microsatellite analysis

Wheat microsatellite (WMS) primer pairs were developed by Roeder et al. (1995) and Roeder et al. (1998). One primer of WMS primer pairs was labeled using either 5-carboxy-fluorescein (5-FAM), 4,7,2′,7′,-tetrachloro-6-carboxy-fluorescein (TET) or 4,7′,2′,4′,5′,7′-hexachloro-6-carboxyrhodamin (HEX). Each PCR reaction contained 50 ng genomic DNA, 10 pmol of each labeled and unlabeled primer, 0.75 U *Taq* DNA polymerase (Qiagen), 2 µl of 10 x PCR buffer containing 15 mM $MgCl_2$, 0.3 mM dNTPs in a total volume of 20 µl. The PCR reaction was carried out in a PE 9600 thermal cycler programmed for 35 cycles at 95°C

for 10 s, the annealing temperature was either 55°C or 60°C according to the primer pairs used for 10 s, and 72°C for 30 s, with a final step at 72°C for 10 min. The PCR products amplified with Hex-labeled primer and 5- FAM- or TET-labeled primer were diluted with water at 1:4 and 1:9, respectively.

The samples were mixed with 0.15 µl GeneScan-500 TAMRA internal size standard (Applied Biosystems) and 0.85 µl formamide dye, denatured at 90°C for 3 min and chilled on ice. Electrophoresis of 36 samples was carried out using 5% denaturing polyacrylamide gel (Long Ranger™, FMC Bioproducts) in 1 x TBE buffer on an ABI prism™ 377 DNA Sequencer (Applied Biosystems) at 1200 V for 1.5 h. ABI collection software version 1.1 was used for raw data collection. Microsatellite fragments were analysed using GENESCAN™ analysis software version 2.1 as described in the user's manuals.

2.4.3 Sequence-tagged site (STS) PCR

PCR amplification using STS primer pairs was carried out in 50 µl reactions containing 75 ng genomic DNA, 0.2 µM of each primer, 0.2 mM dNTPs, 1.5 U of Taq polymerase. Reaction conditions were 1 cycle of 94°C for 2 minutes, 62°C for 30 seconds, 72°C for 1.30 minutes followed by 34 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 1.30 minutes. The last step was 72°C for 10 minutes.

2.5 Marker nomenclature

AFLP marker designations were based on the primer combination used and the fragment sizes estimated accurately with reference to the internal lane standard Gene Scan-500 ROX (Applied Biosystems). The primer combinations are abbreviated according to the list which was provided by KeyGene company and can be accessed in the Grain Genes database (http:///Wheat.pw.usda.gov/ggpages/keygene AFLPs.html). Accordingly, the abbreviations of the primers used can be found in Table 5. Microsatellite loci were designated *Xgwm* followed by a probe number, according to Röder et al. (1998b). Primer pairs were chosen based on their location in the wheat genome. The information of microsatellite primer pairs used in this study can be found in Table 6. All primers were synthesized by INTERACTIVA Biotechnologie GmbH (Ulm, Germany).

Table 5. List of AFLP primer combinations used for screening of the DNA bulks

Code	*Eco*RI+ANN	Code	*Mse*I+CNN	Code	*Mse*I+NN	Code	*Sse*8387I+NN
E 31	E+AAA	M 47	M+CAA	M 12	M+AC	S 12	S+AC
E 32	E+AAC	M 48	M+CAC	M 13	M+AG	S 16	S+CC
E 33	E+AAG	M 49	M+CAG	M 14	M+AT	S 19	S+GA
E 34	E+AAT	M 50	M+CAT	M 15	M+CA	S 20	S+GC
E 35	E+ACA	M 51	M+CCA	M 16	M+CC	S 22	S+GT
E 36	E+ACC	M 52	M+CCC	M 17	M+CG		
E 37	E+ACG	M 53	M+CCG	M 18	M+CT		
E 38	E+ACT	M 54	M+CCT	M 19	M+GA		
E 39	E+AGA	M 55	M+CGA	M 20	M+GC		
E 40	E+AGC	M 56	M+CGC	M 21	M+GG		
E 41	E+AGG	M 57	M+CGG	M 22	M+GT		
E 42	E+AGT	M 58	M+CGT	M 23	M+TA		
E 43	E+ATA	M 59	M+CTA	M 24	M+TC		
E 44	E+ATC	M 60	M+CTC	M 25	M+TG		
E 45	E+ATG	M 61	M+CTG	M 26	M+TT		
E 46	E+ATT	M 62	M+CTT				

Table 6. List of SSR primer pairs used for screening of the DNA bulks

Locus	Chr.	Left Primer Right Primer	Annealing Temperature
Xgwm10	2A	CGC ACC ATC TGT ATC ATT CTG TGG TCG TAC CAA AGT ATA CGG	50°C
Xgwm47	2A	TTG CTA CCA TGC ATG ACC AT TTC ACC TCG ATT GAG GTC CT	60°C
Xgwm95	2A	GAT CAA ACA CAC ACC CCT CC AAT GCA AAG TGA AAA ACC CG	60°C
Xgwm294	2A	GGA TTG GAG TTA AGA GAG AAC CG GCA GAG TGA TCA ATG CCA GA	55°C
Xgwm296	2A	AAT TCA ACC TAC CAA TCT CTG GCC TAA TAA ACT GAA AAC GAG	55°C
Xgwm382	2A	GTC AGA TAA CGC CGT CCA AT CTA CGT GCA CCA CCA TTT TG	60°C
Xgwm473	2A	TCA TAC GGG TAT GGT TGG AC CAC CCC CTT GTT GGT CAC	55°C
Xgwm497	2A	GTA GTG AAG ACA AGG GCA TT CCG AAA GTT GGG TGA TAT AC	55°C
Xgwm512	2A	AGC CAC CAT CAG CAA AAA TT GAA CAT GAG CAG TTT GGC AC	60°C
Xgwm148	2B	GTG AGG CAG CAA GAG AGA AA CAA AGC TTG ACT CAG ACC AAA	60°C
Xgwm257	2B	AGA GTG CAT GGT GGG ACG CCA AGA CGA TGC TGA AGT CA	60°C
Xgwm319	2B	GGT TGC TGT ACA AGT GTT CAC G CGG GTG CTG TGT GTA ATG AC	55°C
Xgwm374	2B	ATA GTG TGT TGC ATG CTG TGT G TCT AAT TAG CGT TGG CTG CC	60°C
Xgwm212	5D	AAG CAA CAT TTG CTG CAA TG TGC AGT TAA CTT GTT GAA AGG A	60°C
Xgwm292	5D	TCA CCG TGG TCA CCG AC CCA CCG AGC CGA TAA TGT AC	60°C

2.6 Development of STS marker

Two AFLP fragments namely S20M13-272 and S20M13-515 which were retrieved from early and late bulked segregants, respectively of Mercia x Mercia(C5912A) mapping population were isolated from dried polyacrylamide gels and cloned for the development of simple PCR markers. Furthermore, allelic variants of these marker loci were cloned from early and late-flowering DNA bulk members, respectively.

2.6.1 AFLP fragment isolation (Schwarz et al., 1999)

Using 5´-Cy5 labeled S20 and unlabelled M13 AFLP primers, AFLP profiles were generated in a conventional sequencing system (model S2, Life Technologies, Inc). After two hours electrophoresis was stopped and the gel was scanned with a fluoroimager (Storm860, Molecular Dynamics). The polymorphisms could be found easily by comparing the patterns from early and late pools. A sharp, clean razor blade was used to excise the relevant bands. The DNA-containing gel piece was placed in a 1.5-ml microcentrifuge tube and rehydrated with 50 µl TE buffer overnight. Two µl of DNA was reamplified using non-selective AFLP primers.

2.6.2 Purification of PCR product

Prior to cloning, PCR products were purified using QIAquick PCR purification kit (Qiagen). Five volumes (100 µl) of Buffer PB was mixed with 1 volume (20 µl) of the PCR sample. QIAquick spin column was placed in a provided 2-ml collection tube. The samples were loaded on the QIA quick column to bind the DNA and centrifuged for 1 min at 13.000 rpm. The flow-through was discarded and the column was placed in the same collection tube. To wash the DNA, 0.75 ml of PE buffer was added and centrifuged for 1 min. The flow-through was discarded and the column was re-centrifuged for 1 min at 13.000 rpm. The column was placed in a clean 1.5 ml microcentrifuge tube. To elute the DNA, 25 µl of ddH_2O was added to the centre of the QIAquick membrane and the column was centrifuged for 1 min at 13.000 rpm.

2.6.3 Cloning of AFLP fragment (Schwarz et al., 1999)

The purified PCR fragments were cloned into pGEM-T Easy vector (Promega). To calculate the appropriate amount of PCR product (insert) to be used for ligation reaction, following equation was used:

$$\frac{\text{ng of vector x size (kb) of insert}}{\text{size (kb) of vector}} \times \text{insert: vector molar ratio} = \text{ng of insert}$$

In these experiments, a 3: 1 insert: vector molar ratio was applied. The 20-µl cloning reaction contained an appropriate amount of PCR product, 50 ng of pGEM T-Easy vector, 3 Weiss units of T4 DNA ligase in 1 x T4 DNA ligase buffer. This reaction was incubated in a refrigerator (4°C) overnight.

2.6.4 Transformation (Hanahan, 1983)

Five microliters of the cloning reaction were then mixed with 100 µl competent cell (XL-1 Blue). The mixture was incubated for 30 minutes on ice. The cells were heat shocked for 90 seconds at 42°C. The tube was transferred immediately to ice and incubated for 5 minutes. 400 µl of SOC medium was added to the bacterial cells and further incubated for 45 minutes at 37°C. The solution was then centrifuged for 3 minutes at 6000 rpm. The supernatant was discarded and the pellet was suspended with 500 µl LB + Ampicillin(120 µg /ml). This procedure was repeated twice and the pellet was suspended with 150 µl LB + Ampicillin(120 µg /ml). This solution was then spread onto LB agar + Ampicillin(120 µg /ml) and incubated overnight at 37 ^{0}C.

2.6.5 Isolation of recombinant plasmids (Birnboim and Doly, 1979)

3 ml of LB medium supplemented with 120 µg Ampicillin/ml was inoculated with a single colony of *E.coli* cells carrying a recombinant plasmid vector, and incubated for 18 hours at 37°C.

Recombinant plasmids were isolated using Boehringer Kit. 1.5 ml of over-night culture was centrifuged for 4 minutes at 6000 rpm. The supernatant was discarded and the pellet was resuspended in 250 µl suspension buffer. After well mixing, 250 µl lysis buffer was added, mixed gently and incubated for 5 minutes at room temperature. After incubation, 350 µl chilled binding buffer was added and the tube was inverted gently 3 to 6 times followed by incubation on ice for 5 minutes. The solution became cloudy and flocky. Centrifugation was done for 10 minutes at 13000 rpm. A compact white pellet will be formed. The supernatant was then transferred into filter tube which have been put inside collection tube followed by centrifugation for 30 to 60 seconds at max speed. The flow through was discarded and 700 µl wash buffer II was added. Centrifugation was done for 30 to 60 seconds at maximum speed. The flow through was discarded and centrifugation was repeated for an additional 30 to 60

seconds to remove residual wash buffer. The collection tube was then discarded and the filter tube was inserted in a clean 1.5 ml reaction tube. 75 µl elution buffer or water was added and centrifuged for 30 seconds to get eluat.

2.6.6 Analysis of recombinant plasmids

Each of recombinant plasmids was digested with *Eco*RI. The 20-µl restriction assay contained 4 µl of DNA, 5 U of appropriate enzyme in 1x restriction buffer and was incubated for 2 hours at 37°C. Plasmids shown to carry the expected fragments were then diluted to be 50 ng/µl.

Another method for recombinant plasmid analysis was applied for plasmids carrying DNA fragment generated from STS primer. Five clones each for the 4 different fragments (*PpdB1*-272early-flowering member, *PpdB1*- 272late-flowering member, *PpdB1* -515early-flowering member and *PpdB1*-515late-flowering member) were inoculated into 5 ml of Luria-Bertani broth and incubated for 18 hours at 37°C. Two ml of overnight culture was transferred into 2 ml reaction tubes. PCR was made with M13 primers using 1 µl of overnight culture. Meanwhile cells were harvested by centrifuging for 4 minutes at 6000 rpm. The supernatant was removed thoroughly with pipette and the pellet was kept in refrigerator waiting for PCR result. From 1.2 % agarose test gel, it was chosen three best clones each for the 4 different fragments. Plasmid DNA from the transformants was isolated using the similar method like written in section 2.6.4

2.6.7 Sequence analysis (Sanger et al., 1977; Murray, 1989)

Sequencing reactions were carried out using BigDye^{TM} Terminator Cycle Sequencing v2.0 (ABI PRISM). The DNA inserts were sequenced from both ends of the inserts, using M13 reverse (5'-GTAAAACGACGGCCAG-3') and M13 forward primer (5'-GTAAAACGACGG CCAG-3'). The sequence reaction was carried out in 10 µl volume containing 3 µl plasmid (150 ng), 4 µl of sequencing reagent premix, 1.25 µl forward/reverse primer (2.5 pmol) and 1.75 µl H_2O. Reactions conditions were 25 cycles of 95°C for 20 seconds, 50°C for 15 seconds and 60°C for 60 seconds.

Before loading on a sequencing gel, PCR products were precipitated using 1 µl of 3 M NaOAc/EDTA and 40 µl of 96 % ethanol and incubated for 15 minutes on ice. Centrifugation was done for 15 minutes at 13000 rpm. The supernatant was discarded and the pellet was washed with 500 µl of 75 % ethanol. After centrifugation for 5 minutes at 13000

rpm, the pellet was air dried. The pellet was then resolved in 2 µl loading dye. The sequencer was run for 7 hours at 51°C and 1680 V.

2.6.8 Primer design

After deleting the sequence data of adaptor and recognition sites of the enzymes used, primer sets were designed using Primer Premier Program. The STS primer were initially utilized to amplify the genomic DNA from early and late-flowering DNA bulk member with PCR conditions as written in section 2.4.3. PCR products were electrophoresed on 1.5 % agarose gels containing 0.5 µg/ml ethidium bromide at 70 V for 45 minutes.

2.6.9 Cloning and sequencing of STS products

Since with the two primer pairs fragments of similar size from both early and late-flowering DNA bulk members were amplified (as detected by agarose gel-based analysis), PCR products amplified with STS primer were cloned again using TA Cloning Kit for Sequencing (Invitrogen). The cloning reaction contained 3 µl of fresh PCR products, 1 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$), 1 µl of H_2O and 1 µl of TOPO vector. The reaction mixture was incubated for 5 minutes at room temperature. The resulting clones were sequenced to survey polymorphisms within the marker fragments.

2.6.10 CAPS analysis

No polymorphism was detected from sequence alignments for the 272-bp AFLP-derived STS marker, while four SNPs and a 20-bp insertion deletion (indel) were detected from 515-bp AFLP-derived STS markers. A CAPS primer was designed for restricting this indel region, so that enable to differentiate early and late-flowering progenies. This The PCR product (50 µl) amplified by 515 bp-AFLP derived STS marker was restricted with *Hin*6I (5 units) and electrophoresed. The conditions for PCR and electrophoresis were the same as described above.

2.7 Linkage analysis

Linkage analysis of all marker loci was performed with the computer program MAPMAKER version 3.0 (Lander et al., 1987) for selfed recombinant inbred lines. Markers were placed with a LOD threshold of 3.0. The Kosambi mapping function was applied to convert recombination into map distances (Kosambi, 1944). The linkage analysis was

performed as an interative procedure, considering first only the undistorted marker loci and adding the distorted markers in a second step. This should help to detect artefactual linkage groups caused by strong distortion. In the first step linkage groups were determined with a likelihood odds (LOD) ratio of 3.0 as a threshold.

The "compare" command calculated the maximum likelihood map for each specified order of markers, and to report the orders sorted by the likelihood of their maps. One sequence can specify more than one order of loci. For example, the sequence "{1 2 3}" specifies the three orders: "1 2 3", "1 3 2", and "2 1 3". Only the 20 most likely orders were reported (by default) by MAPMAKER. The best order was indicated as having a relative *log-likelihood* of 0.0.

The "order" command was then used to find a linear order of the markers on chromosome. Briefly, this command performs the following analyses: (1) it tries to find a small subset of loci (by default, 3 loci), for which a single order is found to be much more likely than any other using a "compare" style analysis; (2) remaining markers which can be mapped to a unique position are added to this order one at a time; (3) at the end, any markers which cannot be mapped to a unique position in the order are mapped into multiple intervals.

Multipoint analysis was used in order to determine the best order of marker loci within a linkage groups. The "ripple" command was conducted to assign exact positions to markers. Marker did not meet the threshold were placed in the interval using the mapmaker "try" command. For the drawing of the map the computer program MapChart 2.1 was used (Voorrips, 2002).

3 Results

3.1 *Vrn-D1* (*Vrn3*) population

A single chromosome recombinant lines (SCRLs) population segregating for *Vrn-D1* gene and derived from the cross between Chinese Spring (CS) and an intervarietal chromosome substitution line in which chromosome 5D of CS was replaced by its homologue from the variety Hope was employed in this study. In this material recombination is restricted to chromosome 5D on which CS carries the vernalization insensitive *Vrn-D1* and Hope the sensitive *vrn-D1* allele. Bulked Segregant analysis (BSA) method was used for identifying polymorphic markers. The bulks were constructed by pooling the same amount of DNA from individuals that have identical phenotypes.

Previous screening of two SSR markers, WMS212 and WMS292, originating from wheat chromosome 5D, and 150 AFLP primer combinations (PCs) between the parental lines CS and CS(Hope5D) and the early-flowering and the late-flowering DNA bulks revealed that only WMS292 was polymorphic (Bäumler, 2000). The analysis of SSR locus WMS292 showed that CS and the *Vrn-D1* bulk carry an allele with 221 bp, the *vrn-D1* bulk an SSR allele with 217 bp, whereas substitution line CS(Hope5D) was heterozygous for both marker alleles (Figure 2).

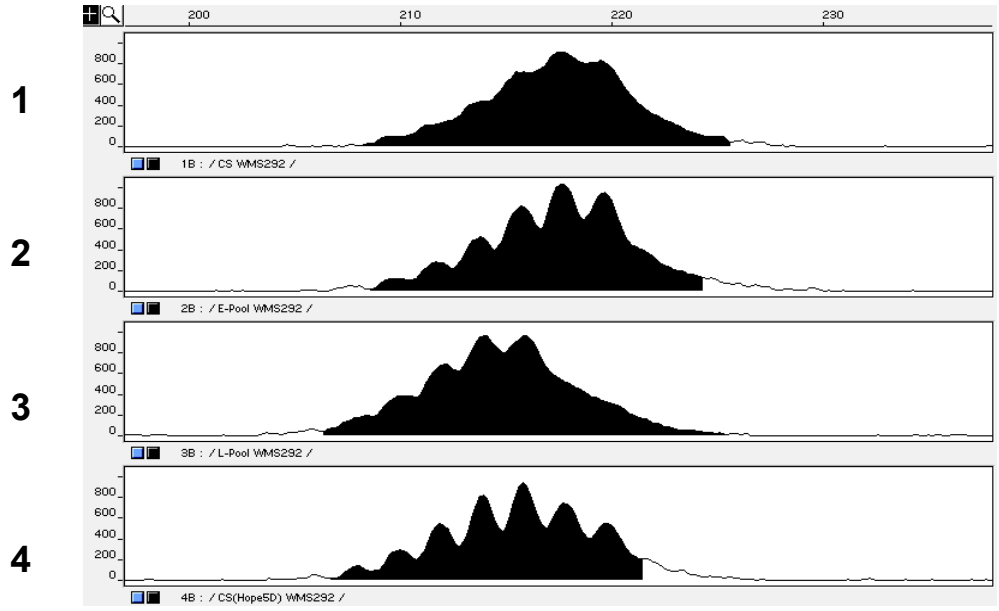

Figure 2**.** Electropherograms of microsatellite profiles from **(1)** CS, **(2)** early-flowering DNA bulk, **(3)** late-flowering DNA bulk and **(4)** CS(Hope5D) after amplification with SSR primer pair WMS292

SSR marker WMS292 was further used to check its linkage to the *Vrn-D1* gene, using the above mentioned segregating SCRL population. Genotyping of 94 individuals showed that WMS292 was closely linked to the *Vrn-D1* gene, with a map distance of 2.81 cM (LOD 19.78). SSR primer set WMS212 generated a fragment of 107 bp in both the bulks and the parental lines, and hence was not useful for further analysis.

To check the specificity of this marker, validation was made using 52 CIMMYT wheat cultivars and 8 tester lines (Triple Dirk), which carry different *Vrn* genes. Six different marker alleles were detected among these cultivars after amplification with WMS292, where the sizes of the amplified fragment ranged from 211 bp to 223 bp. From this validation, it was found that the expected size from CS (221bp) was not an informative marker for *Vrn-D1* gene since one of the tester line, Triple Dirk E, which is proved to carry *Vrn-D1* gene showed a marker allele of 215 bp. The other 7 Triple Dirk lines showed the 211-bp marker allele together with 3 CIMMYT cultivars. The frequency of WMS292 marker alleles among 52 CIMMYT wheats can be seen in Fig. 3.

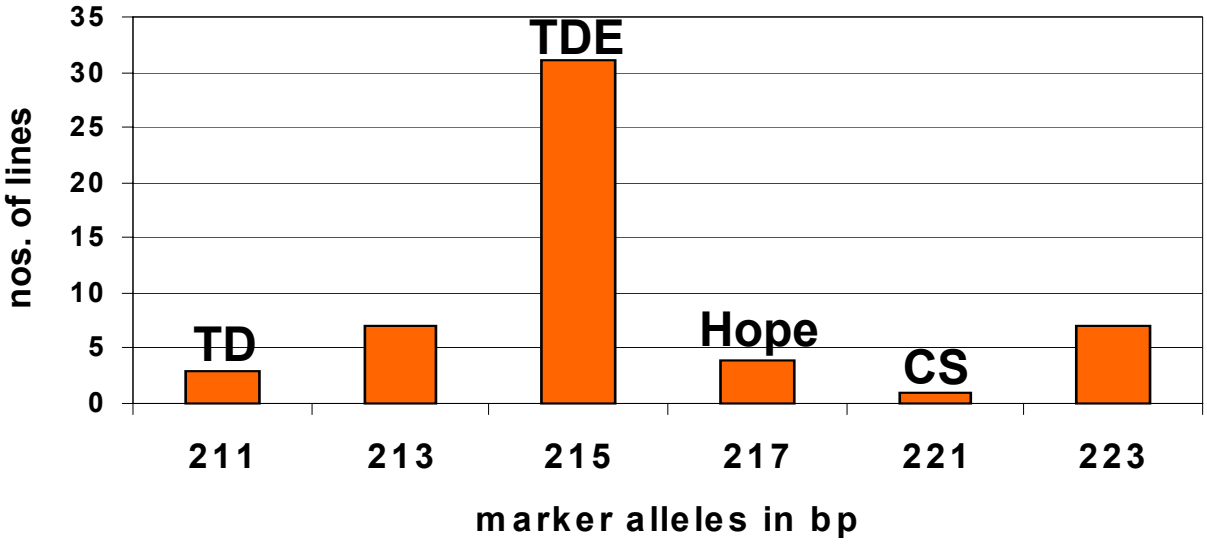

Figure 3. Frequency of gwm292 marker alleles among 52 CIMMYT wheats

Triple Dirk E (TDE) showed a marker allele of 215 bp, while other 7 Triple Dirk (TD) showed the 211-bp marker allele. CS and Hope showed marker alleles of 217 bp and 221 bp, respectively.

In order to find out if there is a correlation of the 215-bp marker allele with the presence of *Vrn-D1* gene, comparisons between allele data from gwm292 and phenotypic data in a set of 36 CIMMYT wheat cultivars were conducted (Table 7).

Table 7. Association of *Xgwm292*-215 marker allele with presence of *Vrn-D1* in CIMMYT wheats

Cross	*Vrn-A1*	*Vrn-B1*	*Vrn-D1*	*Vrn4*	Other *Vrn*	gwm 292
HD2329	*	*	*	*		211
Inqilab91	*	*				**215**
Scan		*	*			**215**
Siete Cerros	*	*				223
Bacanora			*			**215**
CNO79/PRL	*	*				213
Rayon			*			**215**
HE1/2*CNO79	*					213
Star		*				213
Turaco		*	*			**215**
Weaver		*	*			**215**
Baviacora		*	*			**215**
Irena			*			**215**
Seri		*	*			**215**
Turaco/Chil			*			**215**
Pastor			*			215
PGO/Seri//BAU	*					223
Embrapa 16					*	223
Enkoy	*	*				217
Attila			*			**215**
Kauz			*			**215**
Pavon		*	*	*		**215**
VEE #5/Sara		*	*	*		**215**
Chum18//JUP/BJ			*			223
Munia	*					213
Milan /Sha7	*		*			**215**
Don Ernesto		*				213
Nesser	*	*				223
Chilero			*			**215**
Gen*3/PVN			*			**215**
Pitta	*					**215**
Tui		*				213
Chil/PRL		*	*			**215**
Chilero/BUC	*		*			**215**
Prinia	*		*			**215**
PBW343			*			**215**
Chinese Spring			*			221
Triple Dirk E			*			**215**

The *Vrn* classification for these 36 cultivars was done by test crosses (Triple Dirk series) at CIMMYT. For example, a cultivar was categorised to carry *Vrn-D1* gene if all progenies from crossing between this line and Triple Dirk E showed early-flowering character. From this comparison it was found that there were just 4 misclassifications from 36 lines. Two lines that carry *Vrn-D1* (HD2329 and Chum18//JUP/BJ) based on phenotypic character were not scored with 215-bp marker allele, while the other two lines (Inqilab91 and Pitta) that carry v*rn-D1 allele* were scored with 215-bp marker allele from gwm292. In other words, 21 of 23 cultivars detected by WMS292 to carry *Vrn-D1* based on the presence of 215-bp marker allele are carrying *Vrn-D1*. This means that there is a high correlation between the presence of the 215-bp marker allele and *Vrn-D1* configuration. Therefore, SSR marker gwm292 can be regarded as a useful marker for detection of *Vrn-D1* gene.

3.2 *Ppd-B1* (*Ppd2*) population

3.2.1 Identification of microsatellite markers linked to *Ppd-B1*

To map photoperiod response gene on chromosome 2B, a SCRL population derived from a cross between CS, which is photoperiod-insensitive and CS(Marquis2B), which is photoperiod-sensitive was utilized. Four SSR primer pairs, WMS148, WMS257, WMS319 and WMS374 from the short arm of chromosome 2B were used to screen the bulks and parents for polymorphism. Two microsatellite loci, *Xgwm148* and *Xgwm257,* were polymorphic between the two bulks and two parents.

A clear distinction was revealed by WMS148, displaying a 164-bp fragment from early-flowering DNA bulk and CS and a 166-bp fragment from late-flowering DNA bulk and CS(Marquis2B). The pattern of amplification by WMS257 can be seen in Figure 4. The early-flowering DNA bulk showed homozygous marker allele like CS, while the late-flowering DNA bulk showed a heterozygous pattern composed of the 195-bp marker allele from CS and the 197-bp marker allele from CS(Marquis2B).

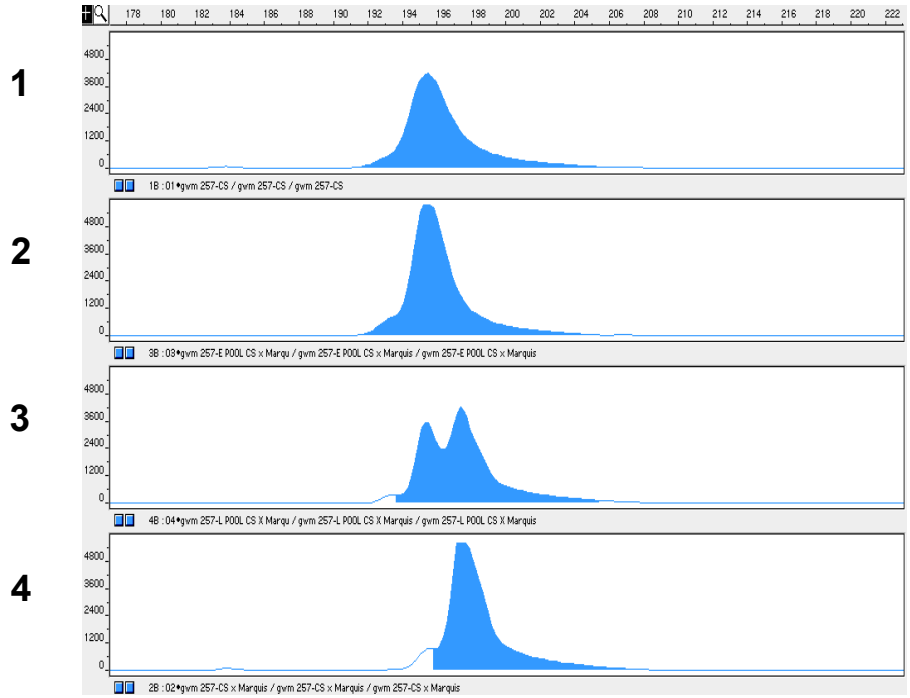

Figure 4. Electropherograms of microsatellite profiles from (**1**) CS, (**2**) early-flowering DNA bulk, (**3**) late-flowering DNA bulk and (**4**) CS(Marquis2B) after amplification with SSR primer pair WMS257

3.2.2 Identification of AFLP markers linked to the *Ppd-B1*

A total of 75 *Sse*8387I+NN/*Mse*I+NN AFLP PCs were used for marker search. Of 7200 amplified marker loci, two AFLPs namely S20M16-337 and S20M16-329 were polymorphic between both the phenotypic bulks and the parental lines. Both markers were linked to *Ppd-B1* in coupling phase. To determine the chromosomal location of these markers, nulli-tetrasomic and ditelosomic lines of CS for chromosomes 2A, 2B and 2D were amplified with the same PC. Figure 5 shows an example how to interpret nulli-tetrasomic analysis of marker S20M16-337. The 337-bp fragment was absent only in N2BT2A, but its presence in the other nulli-tetrasomics confirmed its allocation to wheat chromosome 2B.

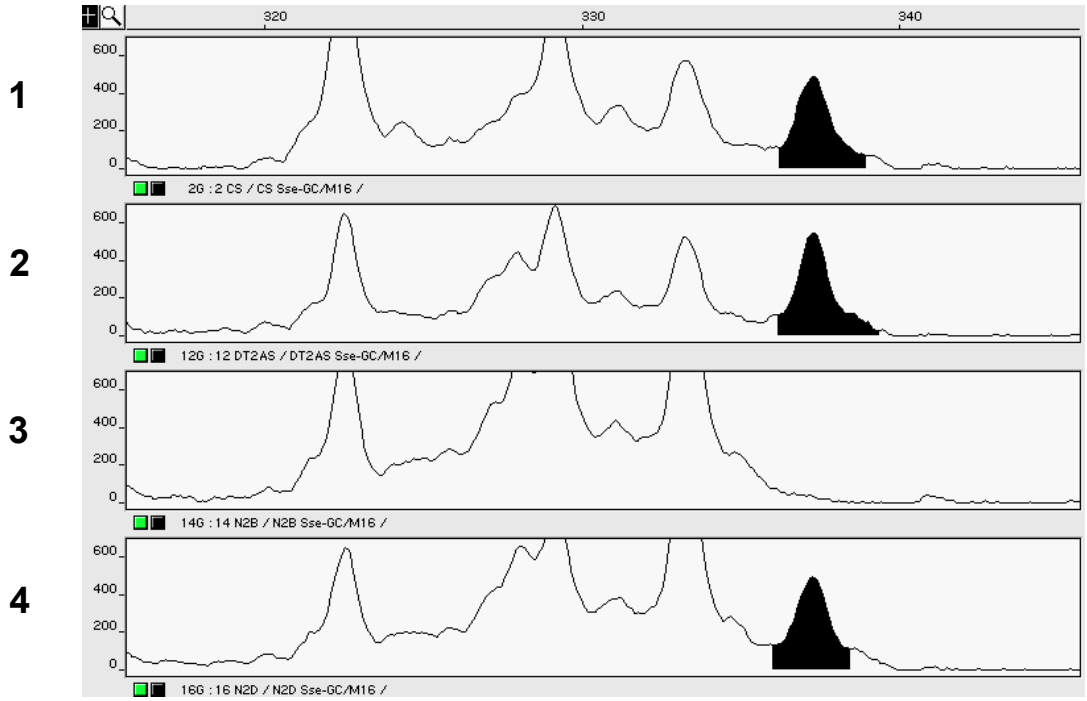

Figure 5. Nulli-tetrasomic analysis of *Ppd-B1* linked AFLP S20M16-337. Amplification of 337-bp fragment (shaded) was found in CS (1), Dt2AS (2) and N2DT2A (4), while it was absent in N2BT2A (3).

3.2.3 Debulking analysis and genetic mapping of *Ppd-B1*

The DNA from individual plants of each DNA bulk was used to confirm the polymorphism of putative markers. In total, 4 markers were found to be linked to *Ppd-B1* gene. Summary of genotypic data of these 4 markers within the early-flowering and late-flowering DNA bulks are shown in Table 8. No deviation from the expected marker allele was detected by gwm257 for early-flowering DNA bulk members. However three of ten late-flowering DNA bulk members showed the marker allele from the early-flowering parental line. In contrast, genotypic data of two AFLP markers revealed good correlation with late-flowering DNA bulk members, but several recombinations were formed for early-flowering DNA bulk members. *Xgwm*148 displayed nearly complete linkage to *Ppd-B1* within the individuals from each bulk, with just one recombination event. Since marker S20M23-329 showed a bias result within early-flowering DNA bulk members, this primer was not included for further analysis.

Table 8. De-bulking of *Ppd-B1* linked molecular markers

	Marker			
	Xgwm257	*Xgwm148*	*XS20M16-337*	*XS20M23-329*
Early entries				
A13.3	195	164	–	–
A20.7	195	164	+	+
A69.5	195	164	+	–
A73.9	195	164	+	+
A74.6	195	164	+	–
A89.10	195	164	+	+
A90.5	195	166	–	+
A97.1	195	164	+	–
A98.2	195	164	+	+
B14.1	195	164	+	–
Late entries				
A42.2	197	166	–	–
A59.9	197	166	–	–
A68.5	195	166	–	–
A77.1	197	166	–	–
A84.6	197	166	–	–
A87.4	197	166	–	–
A88.10	195	166	–	–
A110.5	197	166	–	–
A113.2	195	166	–	–
B5.7	197	166	–	–

Segregation analysis was then continued across the entire population for the three remaining markers. Unlike in the 20 DNA bulk members, more recombination events were detected by gwm148 in comparison to gwm257. The genetic map shows that *Ppd-B1* is flanked by two microsatellite loci *Xgwm257* and *Xgwm148* with map distances of 4.7 cM and 17.3 cM, respectively (Figure 6).

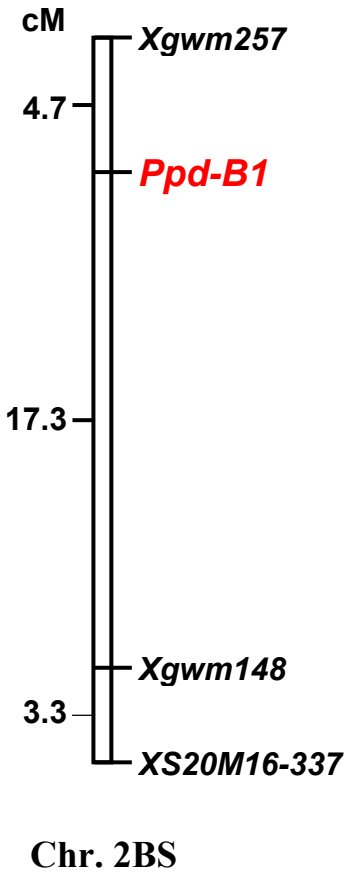

Figure 6. A genetic map around the *Ppd-B1* locus based on the analysis of 72 individuals

3.3 *Ppd-A1* (*Ppd3*) population

3.3.1 Identification of AFLP markers linked to the *Ppd-A1*

A single chromosome doubled haploid (SCDHs) population segregating for *Ppd-A1* and derived from the cross between Mercia, which is photoperiod-sensitive and Mercia(C5912A), which is photoperiod-insensitive was utilized in this experiment. A total of 256 *Eco*RI+ANN–*Mse*I+CNN PCs of 16 possible *Eco*RI+ANN primers and 16 *Mse*I+CNN primers were screened to identify polymorphic AFLP markers between early and late-flowering DNA bulks.

Six PCs were found to be polymorphic between the bulks. However, after validation of putative markers with the DNA of the parents and the members of the bulks, only two of them were classified to represent true polymorphism. AFLP marker E39M60-403 was linked to *Ppd-A1* in repulsion phase and is a 'late-flowering-dominant' marker, generating an additional 403-bp fragment in all of late-flowering DNA bulk members, while it was absent in all of early-flowering DNA bulk members (Figure 7). AFLP marker E37M47-319 was linked to *Ppd-A1* in the coupling phase and is therefore an 'early-flowering-dominant' marker.

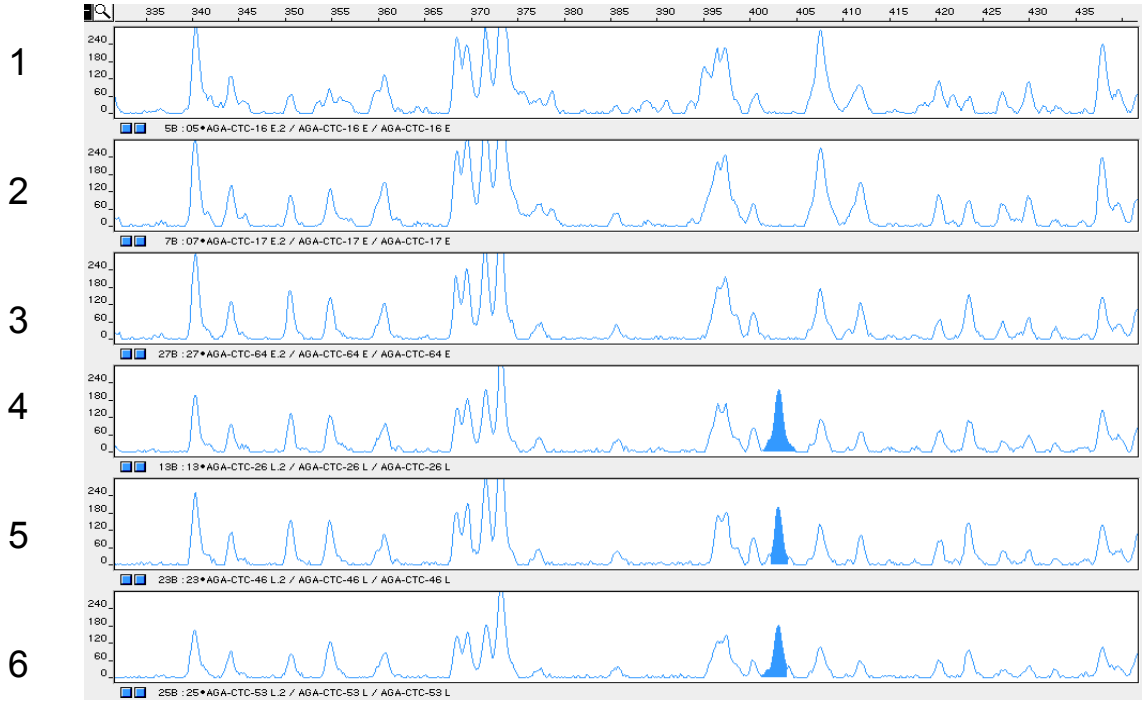

Figure 7. Electropherograms of AFLP profiles in three early-flowering DNA bulk members (1, 2, 3) and three late-flowering DNA bulk members (4, 5, 6) after amplification with PC E39M60. The polymorphic fragment in the size of 403 bp is shown by shaded fragment.

In order to get more polymorphisms, screening was continued with other AFLP enzyme system i.e. *Sse*8387I+NN-*Mse*I+NN. Of 75 PCs tested, 2 polymorphisms were obtained from S20M13. This PC amplified a 272-bp fragment from the early-flowering DNA bulk and a 515-bp fragment from the late-flowering DNA bulk (Figure 8). Further analysis with the DNA bulks members confirmed the complete linkage of putative markers to *Ppd-A1*.

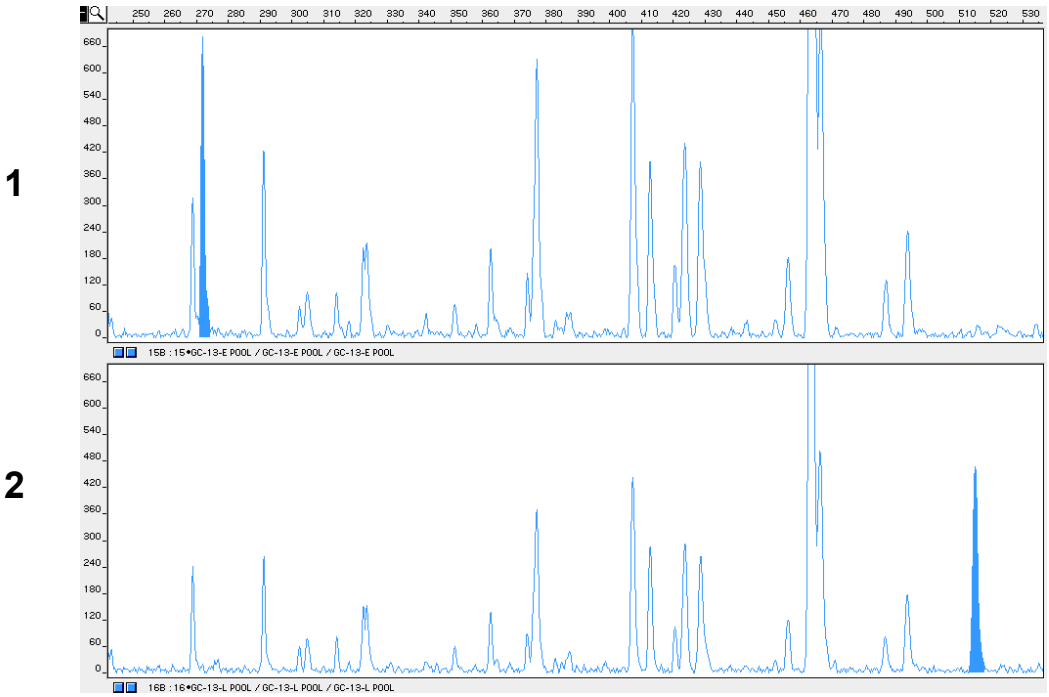

Figure 8. Electropherograms of AFLP profiles showing polymorhic fragments (shaded) with sizes (1) of 272-bp for early-flowering DNA bulk and (2) 515-bp for late-flowering DNA bulk after amplification with S20M13.

3.3.2 Identification of microsatellite markers linked to *Ppd-A1*

Ppd-A1 gene has already been known to be located in the short arm of chromosome 2A. Therefore nine microsatellite primer pairs which are located on chromosome 2A (WMS10, 47, 95, 294, 296, 382, 473, 497 and 512) were used to screen the bulks and two parents for polymorhic markers. All of these microsatellite primer pairs amplified fragments in the same size range as reported by Röder et al. (1998b). However, none of them revealed polymorphism neither between the bulks nor between the parents.

3.3.3 Genetic mapping of molecular markers around *Ppd-A1* locus

All of four AFLP markers were analyzed across the mapping population. No recombination was found between *XS20M13-515* and the *Ppd-A1* gene, indicating that *XS20M13-515* co-segregated with the *Ppd-A1* gene, while *XS20M13-272* was tightly linked and separated from *Ppd-A1* by 2 recombinations, with a map distance of 1.4 cM. This map position is exactly the same like the positions of marker loci *XE37M47-319* and *XE39M60-403* (Figure 9). Interestingly, although all of these markers were tightly linked to *Ppd-A1* gene, none of them were polymorphic between the parents.

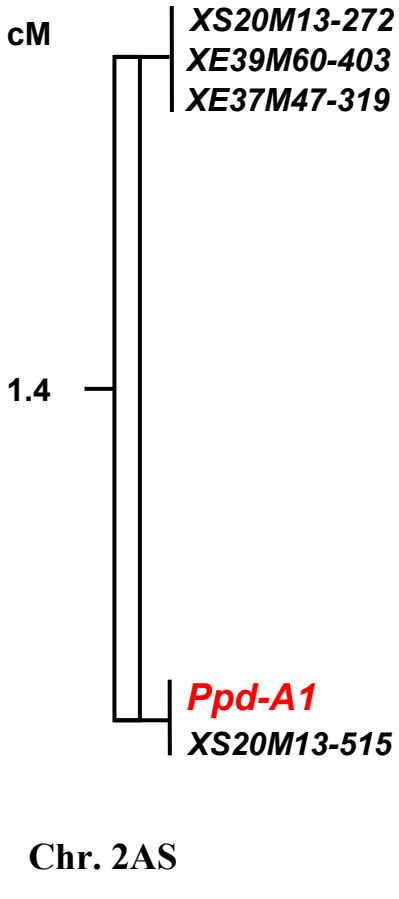

Figure 9. A genetic map around the *Ppd-A1* locus based on the analysis of 74 individuals

3.3.4 Development of STS marker for *Ppd-A1*

The time consuming, multi-step protocol of AFLP technique limits the value of this marker type for molecular breeding purposes. To circumvent this drawback, 2 AFLP fragments linked to *Ppd-A1* (S20M13-272 and S20M13-515) were excised from the polyacrylamide gel, re-amplified with the same PC and subsequently cloned. To confirm the identity of cloned DNA fragments with desired AFLP markers, an improved verification methodology was applied: PCR products from recombinant plasmids (generated with the appropriate AFLP primer combination) were co-electrophorised with the original (multi-locus) AFLP reactions on an automated DNA fragment analyzer.

After sequencing, following primers for amplification of AFLP fragments S20M13-272 and S20M13-515 were synthesized: *Ppd-A1*515_A: 5'-ATA GTG GGA AGT TCA AGG AGT GCA A, *Ppd-A1*515_B: 5'-GTT TGA CCA TGA ATT CCT GTT GTT G-3'; *Ppd-A1*272_A: 5'-AAG CGA TTG TGC GTG TTA CTG T-3', *Ppd-A1*272_B: 5'-ATC CAA ATA ACC AAC TTG AGG C-3'. Since with the two primer pairs fragments of similar size from both early- and late-flowering individuals were amplified (as detected by agarose gel-based analysis), sequencing of marker alleles was carried out to survey for polymorphisms such as single nucleotide polymorphisms (SNPs) or indels (insertions/deletions) within the marker fragments.

After cloning fragments from early and late-flowering individuals, sequence alignments yielded for the 272-bp AFLP derived sequence-tagged site (STS) markers no sequence alterations, whereas for the 515-bp AFLP derived STS markers four SNPs and a 20-bp indel could be detected. The STS marker sizes were 442 bp for early-flowering and 462 bp for late-flowering lines. The indel site (beginning at base position 354) harbours a recognition site for the Hin6I restriction enzyme. On the basis of this sequence information, a cleaved amplified polymorphic sequence (CAPS) marker has been developed and designated as whs2002 (Figure 10).

Agarose test gel of 14 DNA bulk members amplified by whs2002 showed an additional band for late-flowering members of *Ppd-A1* bulk in the size between 300-400 bp which could not be found in early-flowering DNA bulk members. To separate the fragments without the utilization of restriction enzymes, an assay with fluorescently-labelled primers on high resolution gels was established. One of STS primer namely *Ppd-A1*515_A was labeled with 6-Fam at its 5'end. Polyacrylamide gel electrophoresis analysis revealed that the size of the restriction fragment was as expected from the sequence information.

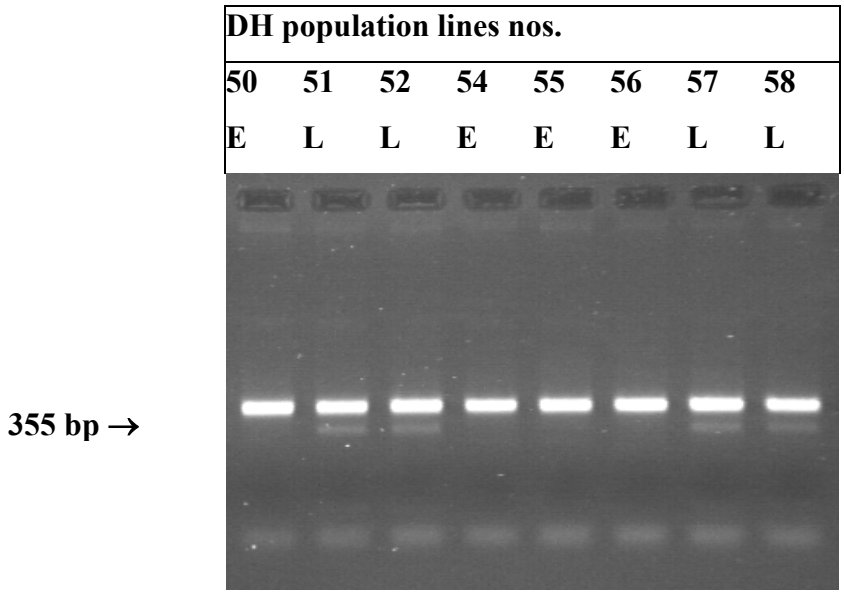

Figure 10. Agarose gel-based analysis of CAPS marker whs2002. An additional band with a size of 355 bp can be seen in late-flowering progenies (L; lines nos. 51, 52, 57, 58), while it is absent in early-flowering progenies (E).

Subsequent scoring of the remaining mapping population revealed that there was no recombination found between CAPS whs2002 and *Ppd-A1*. This means that whs2002 co-segregates with the original AFLP marker S20M13-515.

3.4 Genotyping of 36 CIMMYT lines with markers tightly linked with *Ppd-A1* and *Ppd-B1*

To have an impression of distribution of the markers found to be linked to *Ppd-A1* and *Ppd-B1* genes, 36 CIMMYT lines, which have been confirmed by test crosses made at CIMMYT for the presence of the respective *Ppd* and *Vrn* genes, were amplified by markers gwm257, E39M60-403 and CAPS whs2002 (Table 9).

An interpretation from this table is not easily obtained if the genotypic data of these three markers are treated as the two different markers associated with *Ppd-A1* and *Ppd-B1* separately. It has been known from previous experiment that both of marker E39M60-403 and CAPS marker whs2002 are indicators for late flowering character (*ppd-A1*) in Mercia x Mercia(C5912A) population. So based on the genotypic data from these two markers, it seems that most (30 of 36) of CIMMYT lines tested in this experiment carry *ppd-A1* or having late character in flowering. In contrast, genotypic data of gwm257, an indicator for early flowering lines carrying *Ppd-B1*, revealed that 24 of 36 lines carry *Ppd-B1* (195-bp) or having early character in flowering.

Having known this problem, another approach of analyzing was conducted. Genotypic data from three markers were compared ignoring the fact that each marker correlates to certain *Ppd* gene. Interestingly, there is good correlation between marker allele 195-bp from gwm257 and marker allele 355-bp from CAPS whs2002, while marker allele 197-bp from gwm257 has good correlation with marker allele 403-bp from marker E39M60. This result can be interpreted that CAPS whs2002 which co-segregating with *Ppd-A1* and marker E39M60-403 which is tightly linked to *Ppd-A1* behave like gwm257 which is tightly linked t*o Ppd-B1*. Since it has been known that gwm257 is a specific marker for chromosome 2B of wheat (Röder et al., 1998b), based on the results of this experiment, the location of other two markers should also be in the same chromosome.

For investigation of relationship among these three marker alleles, the marker E39M60-403 and CAPS whs2002 were tested in pools and parents of CS x CS(Marquis2B) population and marker gwm257 was tested in pools and parents of Mercia x Mercia (C5912A) population. The result revealed that these three markers were polymorphic among pools and parents of both populations. However, CAPS whs2002 amplified a 355-bp fragment from early flowering pool and CS in CS x CS(Marquis2B) population, or functions as an early-flowering marker. This means that this CAPS marker behaved in different linkage phase in two different populations. As a consequence this marker is not a diagnostic marker for *Ppd-B1* gene.

Table 9. Genotyping of 36 CIMMYT lines with markers tightly linked with *Ppd-A1* and *Ppd-B1*

Cross	whs2002	gwm257	E39M60-403
HD2329	—	193	—
Inqilab91	355	195	—
Scan	—	197	403
Siete Cerros	355	195	—
Bacanora	—	193	—
CNO79/PRL	—	193	—
Rayon	355	195	—
HE1/2*CNO79	—	195	403
Star	—	197	403
Turaco	355	195	—
Weaver	355	195	—
Baviacora	355	195	—
Irena	355	195	—
Seri	355	195	—
Turaco/Chil	355	195	—
Pastor	—	197	403
PGO/Seri//BAU	355	195	—
Embrapa 16	—	193	—
Enkoy	355	193 &195	403
Attila	355	195	—
Kauz	—	193	—
Pavon	355	195	—
VEE #5/Sara	355	195	—
Chum18//JUP/BJY	355	195	—
Munia	—	197	403
Milan /Sha7	355	195	—
Don Ernesto	355	195	—
Nesser	355	195	—
Chilero	355	201	—
Gen*3/PVN	355	195	—
Pitta	355	195	—
Tui	355	195	—
Chil/PRL	355	195	—
Chilero/BUC	—	197	403
Prinia	355	195	—
PBW343	—	193	—

Further testing showed that nearly all markers found in Mercia x Mercia(C5912A) population could be mapped in CS x CS(Marquis2B) population and vice versa. The only marker that could not be mapped in Mercia x Mercia(C5912A) population was gwm148.

An additional prove for the location of CAPS whs2002 was given by nulli-tetrasomic analysis. Agarose test gel of Dt2AS, N2BT2A, N2DT2A and CS amplified by STS Ppd515 and followed by digestion with *Hin*6I revealed that two bands like in Figure 10 were amplified from Dt2AS, N2DT2A and CS. The 355-bp band was missing from N2BT2A confirming its allocation to wheat chromosome 2B. Based on this findings, it was concluded that both populations should carry the same gene i.e. *Ppd-B1*.

3.5 Mercia x Mercia(CS2B) population

3.5.1 Identification of AFLP markers linked to *Ppd-B1*

In order to get comprehensive genetic mapping data of phenotypic bulks from *Ppd-B1*, third population, consisting of 89 SCDH population from the cross between the photoperiod-sensitive Mercia and substitution line Mercia(CS2B), which is photoperiod-insensitive was studied. Of 183 *Eco*RI+ANN–*Mse*I+CNN AFLP PCs screened to identify polymorphism between early-flowering and late-flowering DNA bulks, 21 PCs were informative between the bulks (Table 10). Six of these markers were linked in repulsion phase, three of them were linked in coupling phase and the remaining 12 markers amplified either two or three fragments in both of the bulks.

Ten PCs were chosen to check their linkage to the *Ppd-B1* gene. The criterions used for this selection were:

a. Good reaction quality.
b. Low number of recombinants in debulking analysis.
c. Polymorphic in other two populations: Mercia x Mercia(C5912A) and CS x CS (Marquis2B)

These 10 PCs produced 1243 clearly scorable fragments. The number of fragments per PC varied from 90 to 160 (average 124.3) and the number of polymorphisms ranged from 1 to 4 (average 1.9). Since one of the markers (E41M56-164) was unlinked to *Ppd-B1*, 18 markers were further used. Together with one additional AFLP marker from other two populations (S20M13-272), a total of 19 AFLP markers for this population were obtained.

Table 10. Summary of AFLP primer combinations used to amplify polymorphic fragment between early and late-flowering DNA bulk of Mercia x Mercia(CS2B) population. PCs with asterisks (*) were used for mapping across the population.

			Fragment Size (bp)	
Primer combination	*Eco*RI primers	*Mse*I primers	Early-flowering DNA bulk	Late-flowering DNA bulk
E36M52*	E-ACC	M-CCC	97, 268	112, 161
E36M54*	E-ACC	M-CCT	98	312
E36M55	E-ACC	M-CGA	—	195
E36M57	E-ACC	M-CGG	505	405
E36M60	E-ACC	M-CTC	119	120
E40M52	E-AGC	M-CCC	241	166
E40M62	E-AGC	M-CTT	246	—
E32M56	E-AAC	M-CGC	174	222
E32M60	E-AAC	M-CTC	—	196
E38M53*	E-ACT	M-CCG	146	—
E41M48*	E-AGG	M-CAC	234	—
E41M51	E-AGG	M-CCA	290	—
E41M54*	E-AGG	M-CCT	410	—
E41M56*	E-AGG	M-CGC	—	164
E41M58*	E-AGG	M-CGT	236	—
E39M60*	E-AGA	M-CTC	121	403
E39M62	E-AGA	M-CCC	—	265
E42M53	E-AGT	M-CCG	280	431
E35M52	E-ACA	M-CCC	86	—
E37M60*	E-ACG	M-CTC	179,287	76
E37M47*	E-ACG	M-CAA	319	320, 385

3.5.2 Integration of SSR marker around the *Ppd-B1* locus

Unlike in the first two populations, *Xgwm257* could not be mapped in this population owing to the absence of polymorphism between the pools and parents. However, gwm148 which is monomorphic in Mercia x Mercia(C591) population, could be mapped across this population like in CS x CS(Marquis2B) population. The difference is marker locus *Xgwm148.1* was not polymorphic in Mercia and Mercia(CS2B) population. However, an additional 148-bp fragment (*Xgwm148.2*) was amplified in Mercia and late-flowering DNA bulk, while this fragment was absent in Mercia(CS2B) and early flowering DNA bulk.

3.6 Segregation analysis of markers and the photoperiod insensitive gene *Ppd-B1* in three segregating populations

Table 11, 12 and 13 summarize the segregation analysis for the *Ppd-B1* locus and molecular markers in Mercia x Mercia(C5912A), CS x CS(Marquis2B) and Mercia x Mercia(CS2B), respectively. In the population Mercia x Mercia(C5912A), 74 DH plants were evaluated for phenotypical segregation of early and late-flowering character (Table 11). A total of 46 plants showed late character, and 28 plants revealed early character. (χ^2=4.38). This frequency distribution is exactly the same as shown by another six markers. This means that both of *Ppd-B1* and six markers in this population deviated slightly from the expected 1:1 ratio at a significant threshold $\alpha = 0.05$.

Table 11. Segregation analysis for the *Ppd-B1* locus and molecular markers in SCDH population from the cross Mercia x Mercia(C5912A)

Gene or markers	Number of Plants	Observed number		Expected Ratio	χ^2	P
		X_1X_1[a]	X_2X_2			
Ppd-B1	74	46	28	1:1	4.38	0.036
E39M60-403	74	46	28	1:1	4.38	0.036
S20M13-272	74	46	28	1:1	4.38	0.036
S20M13-515	74	46	28	1:1	4.38	0.036
Ppd-515	74	46	28	1:1	4.38	0.036
gwm257	74	46	28	1:1	4.38	0.036
E37M47-319	74	46	28	1:1	4.38	0.036

The observed segregation of 32 early flowering and 42 late flowering plants in CS x CS(Marquis2B) population corresponded to a 1:1 segregation (χ^2= 0.89) as expected for the segregation of a single dominant gene (Table 12). The statistical analysis also showed that all of markers in this population segregated normally following a 1:1 segregation.

Table 12. Segregation analysis for the *Ppd-B1* locus and molecular markers in SCRL population from the cross CS x CS(Marquis2B)

Gene or markers	Number of Plants	Observed number		Expected ratio	χ^2	P
		X_1X_1[a]	X_2X_2			
Ppd-B1	72	32	40	1:1	0.89	0.345
gwm257	70	34	36	1:1	0.06	0.806
S20M16-337	71	34	37	1:1	0.13	0.718
Ppd-515	71	35	36	1:1	0.01	0.920
E39M60-403	72	35	37	1:1	0.06	0.806
S20M13-272	72	34	38	1:1	0.22	0.639
E38M53-147	72	35	37	1:1	0.06	0.806
E38M53-161	72	32	40	1:1	0.89	0.345
E36M54-312	72	35	37	1:1	0.06	0.806
E41M58-235	70	32	38	1:1	0.51	0.475
E37M60-287	72	33	39	1:1	0.50	0.480
E41M48-233	70	27	43	1:1	3.66	0.056
Gwm 148.1	72	34	38	1:1	0.22	0.639

Of 89 SCRL plants observed for segregation between early and late flowering in Mercia x Mercia(CS2B), 52 plants showed late character, and 37 plants revealed early character (Table 13). This frequency distribution fitted a 1:1 segregation ratio (χ^2= 2.53) further supporting the finding from Table 12 that *Ppd-B1* is conferred by a single dominant gene. Only seven markers deviated slightly from the expected ratio 1:1 ratio at a significant threshold $\alpha = 0.05$, while the remaining thirteen markers corresponded to a 1:1 segregation.

Table 13. Segregation analysis for the *Ppd-B1* locus and molecular markers in SCDH population from the cross Mercia x Mercia(CS2B)

Gene or markers	Number of Plants	Observed number		Expected ratio	χ^2	P
		X_1X_1[a]	X_2X_2			
Ppd-B1	89	52	37	1:1	2.53	0.112
E39M60-191	89	55	34	1:1	4.96	0.026
E39M60-403	89	49	40	1:1	0.91	0.340
E37M60-76	88	54	34	1:1	4.06	0.044
E37M60-179	88	53	35	1:1	4.06	0.044
E37M60-287	88	53	35	1:1	3.25	0.071
E37M47-319	89	43	46	1:1	0.10	0.752
E37M47-320	89	54	35	1:1	4.06	0.044
E37M47-385	89	53	36	1:1	3.25	0.071
E41M48-233	89	52	37	1:1	2.53	0.112
E38M53-146	89	52	37	1:1	2.53	0.112
E36M54-98	89	57	32	1:1	7.02	0.008
E36M54-312	89	54	35	1:1	4.06	0.044
E41M58-235	89	50	39	1:1	1.36	0.244
S20M13-272	89	48	41	1:1	0.55	0.458
E41M54-410	89	54	35	1:1	4.06	0.044
E36M52-97	89	52	37	1:1	2.53	0.111
E36M52-112	89	50	39	1:1	1.36	0.244
E36M52-161	89	53	36	1:1	3.25	0.071
E36M52-268	89	53	36	1:1	3.25	0.071
gwm 148.2	89	53	36	1:1	3.25	0.071

3.7 Comparison of genetic maps around *Ppd-B1* from three populations

Since it has been known that all of three populations carrying the same gene (*Ppd-B1*), markers within these three populations were exchanged and final genetic linkage maps can be seen in Figure 11. In population I (Mercia x Mercia(C5912A)), 6 molecular markers consisting of 4 AFLP, 1 SSR and 1 STS marker were mapped in an interval of 1.4 cM. Four markers were completely linked to each other and separated from *Ppd-B1* by 1.4 cM, while another two markers also formed a cluster and co-segregated with this gene (Figure 11, population I).

Nine AFLP, one STS, and two SSR markers were mapped in a range of 51.1 cM in population II (CS x CS(Marquis2B)). Four markers which were mapped in population I could also be mapped in this population (Figure 11, population II). Although the order of these markers in both populations is still the same, the map distance among them is different. Three marker loci (*XS20M13-272*, *XE39M60-403*, *Xgwm257*) which co-segregated together in population I, could be resolved in population II with distances of 2.2 and 1.4 cM, respectively.

The STS marker which co-segregated with *Ppd-B1* in population I was also mapped 5.4 cM distal to *Ppd-B1* in population II. Marker *XE37M47-319* which is a common marker between population I and III and linked to *Ppd-B1* in coupling phase could not be mapped in population II since CS(Marquis 2B) also amplified the same 319-bp fragment. A gap of 17.3 cM can be seen between *Ppd-B1* and the closest proximal marker locus *Xgwm148.1*.

In population III (Mercia x Mercia(CS2B)), 1 SSR marker and 19 AFLP markers were mapped in an interval of 28.6 cM (Figure 11, population III). Seven markers of them were also mapped in population II but just three markers were common in comparison to population I. The position of marker *Xgwm148* in population II and III could not be compared since marker gwm148 amplified 2 different marker loci in these two populations. Second marker locus of *Xgwm148.2* was mapped 5.3 cM proximal from *Ppd-B1* and co-segregating together with other 4 AFLP markers in population III. Unlike in population I and population II, CAPS marker whs2002 and microsatellite marker gwm257 could not be used for linkage analysis owing the absence of polymorphism between early and late progenies of this cross.

Other interesting phenomenon can be observed is the difference in map distance between common markers. In general the recombination frequency between each marker in population II is higher in comparison to markers in population III. In population III, the distance between marker *XE38M53-146* and the gene *Ppd-B1* was 3.6 cM, compared with 25.7 cM in population II. This is a 7-fold difference of recombination frequency. The next common proximal markers from *XE38M53-146* (*XE37M60-287* and *XE41M58-235*) in populations II and III showed nearly the same map distances among each other. The marker interval *XE41M58-235* - *XE41M48-233* covers approximately 10.5 and 1.1 cM in crosses II and III, respectively. This represents nearly a 10-fold difference in recombination frequency in the same genetic interval between these two populations.

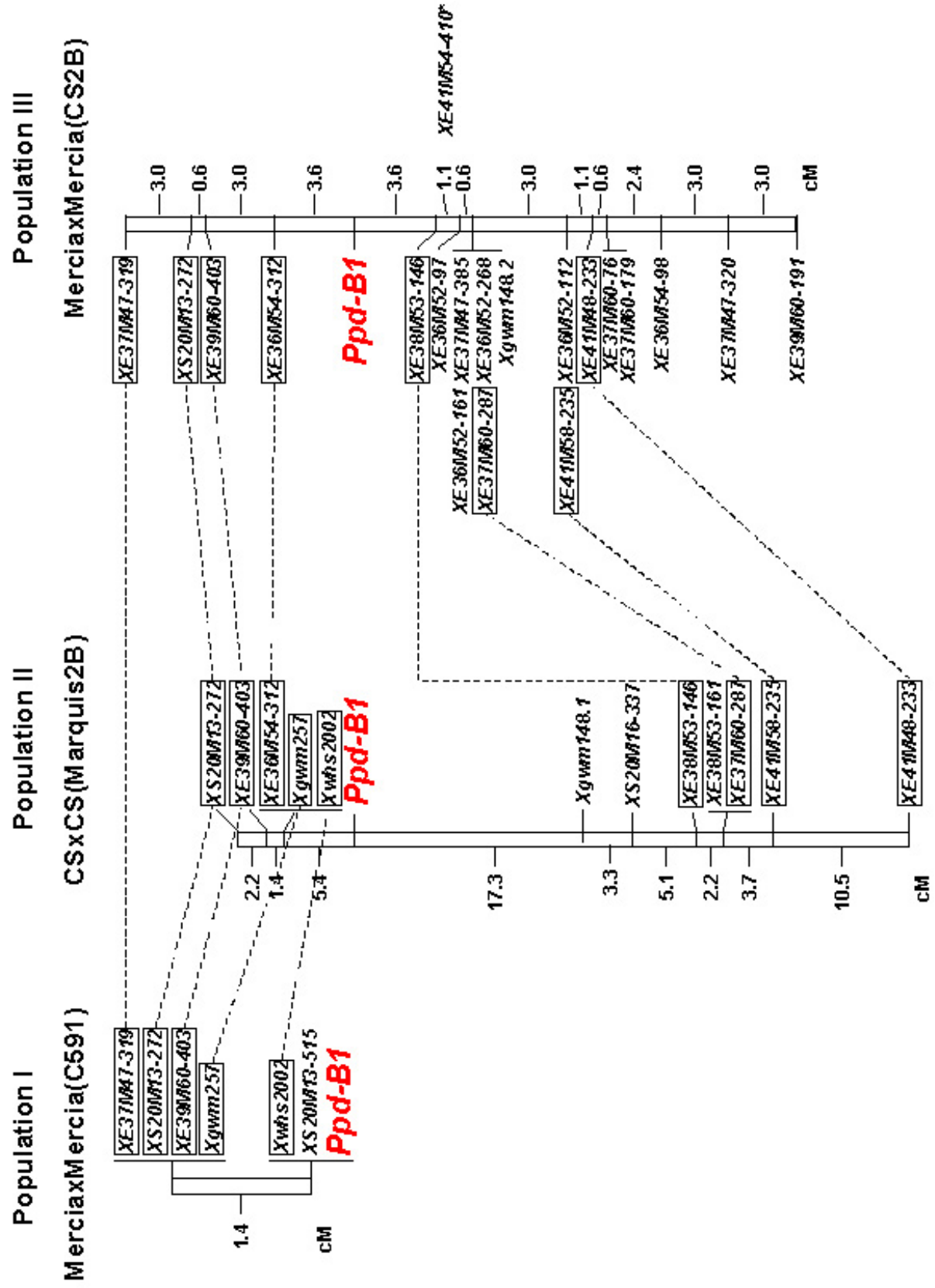

Figure 11. Genetic linkage maps of the *Ppd-B1* on chromosome 2BS of 3 different wheat populations

3.8 Physical mapping of molecular markers

By comparing deletion break points and the markers mapping within deletion intervals on the short arm of chromosome 2 B, a physical map that consists of nine deletion intervals and defined by 10 breakpoints was constructed (Figure 12). This Figure also shows 2 examples (*XE37M60-287* and *XS20M13-272*) how to interpret electropherograms for physical mapping.

Since these deletion lines were developed in CS background, only the markers which were also amplified in CS could be mapped. Of 13 markers linked to *Ppd-B1* gene in coupling phase, nine markers could be mapped physically in deletion lines of chromosome 2BS. The remaining four markers were amplified in all deletion lines. The markers were concentrated in the distal regions and virtually absent from proximal regions. Three markers (*Xwhs2002*, *Xgwm257*, *XS20M13-272*) were mapped distal to fraction length 0.83, while the other six markers were mapped in the largest deletion interval between deletion lines 2BS-3 and 2 BS-6. There was no marker mapped in between this deletion interval.

The markers mapped in deletion intervals on short arm of chromosome 2B indicated that the linear order of both genetic and physical maps is identical. Eleven of 20 markers and 8 of 12 markers of the genetic maps of population II and III, respectively, encompassing a map distance of 13.1 and 35.3 cM, respectively, correspond to the distal region (0.56-0.87) of the physical map.

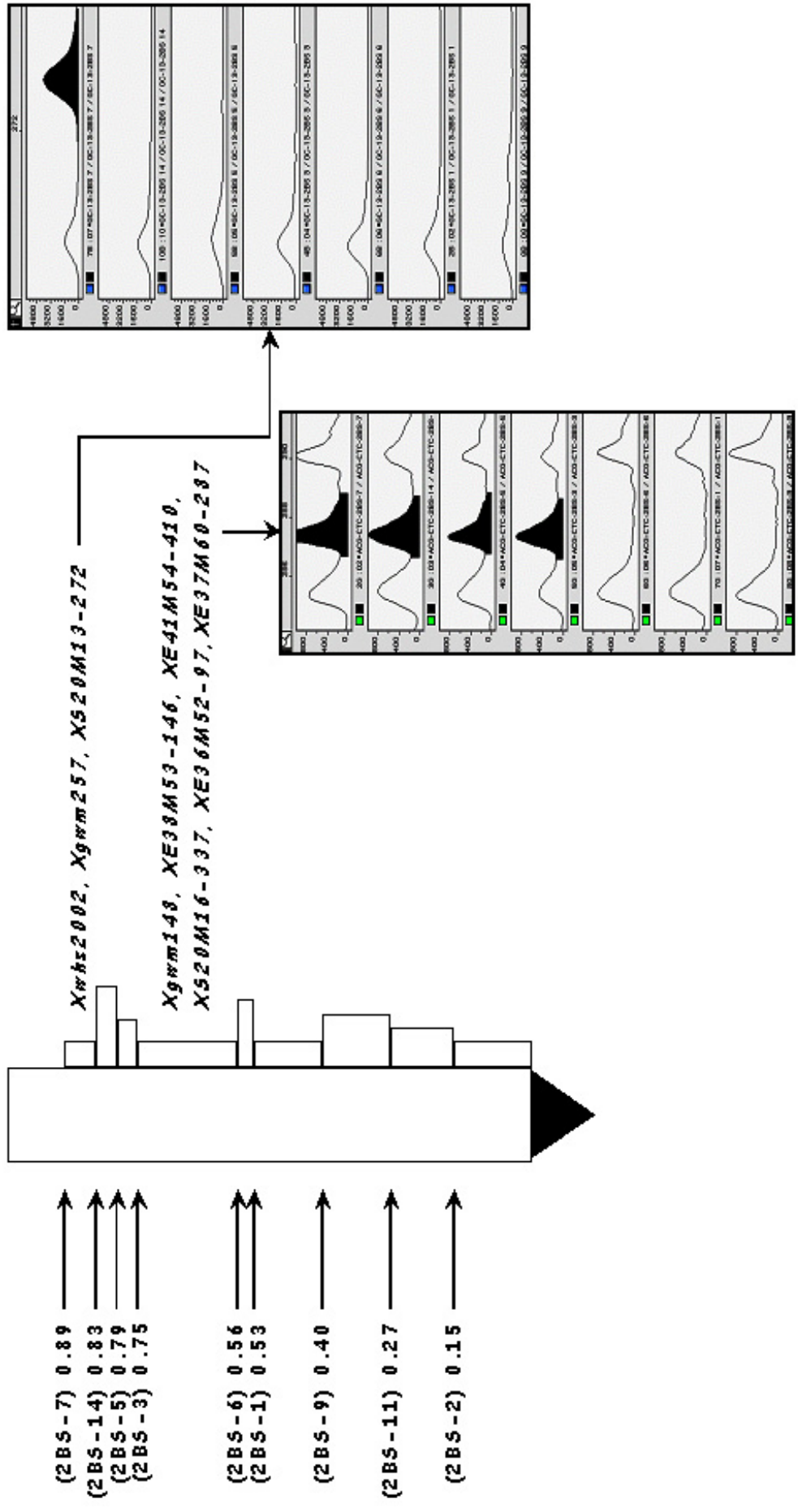

Figure 12. Physical map for chromosome 2BS of wheat. Fraction lengths (FL) and deletion stock numbers are indicated on the left of chromosome. Marker names and corresponding locations on the physical map are indicated on the right side of chromosome.

4 Discussion

4.1 The approach used for this study

Within the cereals, the generation of genetic maps has been slower in hexaploid wheat compared with diploid barley and rice. Some of the difficulties associated with molecular studies in wheat have been the low levels of intraspecific polymorphism and the large number of linkage groups (Nelson et al. 1995a, b; Marino et al., 1996). Ideally, a comprehensive map of the wheat genome would be required for complete analysis but this is time consuming to develop. An alternative option to increase the map density is the use of techniques such as bulked segregant analysis (BSA) (Michelmore et al., 1991). BSA involves the screening of two bulked DNA samples derived from contrasting segregants in a single population, with the aim of identifying polymorphic markers between the bulks. Linkage between polymorphic markers and the targeted locus or trait can be confirmed by analysing the markers on the whole population from which the bulks were constructed.

Among current popular DNA markers, RFLPs and RAPDs have been shown to detect only low levels of polymorphism in wheat (Chao et al., 1989; Devos and Gale, 1992). In contrast, the AFLP technique (Vos et al., 1995) has the capacity to assay a much greater number of loci for polymorphism than other currently available PCR-based techniques, and is suitable for detailed mapping exercises. Furthermore, AFLP loci are distributed throughout the genome of *T. aestivum* (Huang et al., 2000a; Hazen et al., 2002). Similarly, microsatellites can be profitably utilized in wheat not only for detecting polymorphism and tagging genes (Prasad et al., 1999; Roy et al., 1999; Huang et al., 2000b) but also for genotype identification and for estimation of genetic diversity (Prasad et al., 2000). Data on physical mapping of microsatellites on group 2 chromosomes using deletion stocks also confirmed that the microsatellites are not physically clustered in specific regions of the wheat chromosome (Röder et al., 1998a), so that the microsatelllite markers should prove useful for complete coverage of the wheat genome.

The use of AFLP and SSR markers in combination with BSA in this study was proved to be effective. Taking advantage that microsatellite markers are locus-specific, screening of the DNA-bulks could be done quickly and conveniently by choosing microsatellite markers specific to certain wheat chromosomes that contain the gene of interest. However, the current availability of wheat microsatellites still restricted the application of this technique for efficient genotyping, resulting in just a few markers around the target gene. This withdraw

was covered by the use of AFLP technique. Although more efforts and times were needed to find AFLP markers associated with gene of interest, the large amounts of the possible AFLP primer combinations contributed in finding more markers linked to the gene of interest. Therefore, the combination of these two techniques provided valuable approach for rapidly identifying markers linked to the trait of interest.

4.2 Genetics of vernalization gene

Vernalization, the induction of flowering by prolonged exposure to low temperatures, is a major determinant of flowering time and growth habit type in plants. Genes controlling vernalization requirement in hexaploid plant regulate the physiological development and environmental adaptation of the crop, thereby influencing grain yield potential (Barett et al., 2002). In common wheat, three major homoelogous genes concerning vernalization requirement have been identified, i.e. *Vrn-A1*, *Vrn-B1*, *Vrn-D1* on chromosome 5A, 5B and 5D, respectively (Law et al., 1976; Maystrenko, 1980; Galiba et al., 1995; Nelson et al., 1995, Korzun et al., 1997; McIntosh et al., 1998). The degree of requirement to complete heading depends on the *Vrn* genotype. For example, *Vrn-A1* does not require vernalization treatment at all, whereas *Vrn-B1* and *Vrn-D1* require vernalization for 15-30 days, and winter type wheats recessive for all of these genes require 45-60 days for heading (Maystrenko, 1987; Kato, 1988).

The cultivar Hope was reported to be a carrier of dominant *Vrn-A1* and *Vrn-B1*. This was confirmed with the *Vrn* genotypes identified in the spring lines selected from the cross of Hope and Triple Dirk C (Stelmakh, 1998). Law et al. (1966), who worked with several substitution lines for chromosome 5A and 5D, also mentioned that in contrast to CS(Hope 5D), subsitution line CS(Hope5A) is much earlier in ear emergence time than CS. They concluded that *Vrn-A1* is therefore likely to be identical to the gene located on Hope chromosome 5A. From these experiments, it seems that cultivar Hope possess the haploid genotype of *Vrn-A1Vrn-B1vrn-D1*.

Pugsley (1972) has indicated that the spring growth habit of CS is determined by a single dominant gene for vernalization response, *Vrn-D1*. Since nullisomic 5D of CS is the only nullisomic to give both winter habit and extreme lateness in ear emergence (Sears, 1954), then *Vrn-D1* must be located on 5D. In other words, cultivar CS possesses the haploid genotype of *vrn-A1vrn-B1Vrn-D1*. Therefore the use of SCRL population derived from the cross between CS and CS(Hope 5D) for studying molecular markers associated with *Vrn-D1*

gene was a right decision, since the recombination is restricted to chromosome 5D on which CS carries the vernalization insensitive *Vrn-D1* and Hope the sensitive *vrn-D1* allele.

The use of precise genetic stock such as SCRL for genetic analysis of QTL or major genes was suggested by Snape et al. (2001b). By the use of these lines the precise location of QTL or major gene can be obtained because the background genetic variation is effectively eliminated and the trait variation is only influenced by alleles segregating between the single pair of parental chromosomes and associated environmental effects.

Using SCRL population derived from the cross between CS and CS(Cheyenne5D), Snape et al. (2001b) mapped *Vrn-D1* distally on the long arm of chromosome 5D and closely linked to the microsatellite markers *Xgwm212* and *Xgwm292*. The distance between these two markers was just 0.1 cM, in which *Xgwm212* and *Xgwm292* were separated from *Vrn-D1* by 3.3 and 3.4 cM, respectively.

More studies on marker validation are needed before they are extended to applied plant breeding. Marker validation is based on the identification of markers that can be used in different genetic backgrounds (Langridge and Chalmers, 1998; Gupta et al., 1999). Therefore the use of the same microsatellite markers in different genetic background in this experiment can be regarded as an assay to test the specificity of these markers. The failure of marker gwm212 to show polymorphism between both parents and pools of CS x CS(Hope5D) population should be interpreted as this marker is just a specific marker for tagging the presence of *Vrn-D1* in CS x CS(Cheyenne5D) population and not a common diagnostic marker, at least in CS x CS(Hope5D) population.

Results of current experiment with microsatellite marker gwm292 revealed that this marker is closely linked to *Vrn-D1*. Based on this result, it was thought that 221-bp marker allele from CS would be an informative marker allele for tagging *Vrn-D1*. However, genotyping of 36 CIMMYT cultivars together with CS and Triple Dirk E (TDE) as references showed that CS is the only *Vrn-D1* carrying cultivar showing the 221-bp marker allele, while most of *Vrn-D1* carrying cultivars showed the 215-bp marker allele, which was the same as shown by TDE. Since both CS and TDE have been proved to carry *Vrn-D1*, the only possible explanation should be that there are at least two markers alleles (215 bp and 221 bp) at *Vrn-D1* locus to be used for marker assisted breeding depending on the genotype from where *Vrn-D1* was transmitted. Further experiment with larger number of samples including cultivars carrying *Vrn-D1* allele from CS should be done to test this hypothesis.

A study of the global *Vrn* gene distribution supports the hypothesis that *Vrn* loci have different adapted values. The majority of the stocks from the zones of middle latitude seemed

to be carriers of dominant *Vrn-A1* and *Vrn-B1*. When shifting toward the equator, the frequency of dominant *Vrn-D1* increased progressively (Africa, South Asia, Latin America) associated with decreasing *Vrn-A1* and *Vrn-B1* incidence (Stelmakh, 1990). This non-random distribution of three *Vrn* genes was also observed by Iwaki et al. (2000) who studied ecogeographical differentiation of *Vrn* genotypes in East Asia (China, Korea and Japan). They found that *Vrn-D1* was most widely and frequently found among the four genes, followed by *Vrn-A1*, *Vrn-B1*, and *Vrn-4*.

Following these informations, Kato et al. (2001) studied the influence of spring habit gene, *Vrn-D1*, on heading time in wheat. Accordingly, recombinant inbred lines (RIL) with or without *Vrn-D1* gene were produced from F_2 plants of the cross between 'Nanbukomugi' and 'Nishikazekomugi', non-carrier and carrier cultivars of this gene, respectively, and their heading time was examined at different sites in Japan. The results from Fukui fields showed that the average heading time of the early lines was 13 days earlier than that of the late lines. The early-heading lines, however, matured 6 days earlier than the late heading lines, on average. These results were statistically significant different and could be verified at different sites (Kurashiki, Tsukuba and Sapporo) in Japan. This clearly indicates that the *Vrn-D1* gene is necessary to give wheat plants an early heading trait under the environmental conditions in those areas.

An additional advantage of using *Vrn-D1* genotypes has been reported by Stelmakh (1998). Comparing different effect values of *Vrn-A1*, *Vrn-B1*, *Vrn-D1* genes in relation to heading date, plant height and yield components, he found that varieties possessed *Vrn-D1* could adapt to stress conditions such as high temperature and drought at grain filling stage and achieved high grain yield.

Recent studies in the last five years conducted in molecular markers and mapping fields concentrated more on *Vrn-A1* and *Vrn-B1* genes. *Vrn-A1* gene has been mapped physically using wheat deletion lines by Sutka et al. (1999). The comparative mapping of this *Vrn-A1* region with rice has also been conducted by Sarma et al. (1998). Research of *wec* (wheat embryo cold) treatment genes associated with heading factors was also carried out by Shindo et al. (2002) using near isogenic lines of *Vrn-A1*. Several new studies conducted for *Vrn-B1* gene involved identification of AFLP and microsatellite markers for *Vrn-B1* (Barrett et al., 2002), development of dCAPS markers (Iwaki et al., 2002), microsatelllite monitoring of recombination around the *Vrn-B1* locus during early backcross breeding (Salina et al., 2003) and mapping of *Vrn-B1* in relation to *Fr-B1* (Toth et al., 2003). The only studies for *Vrn-D1* gene were just genetic mapping done by Snape et al. (2001) and Shindo et al. (2003), in which

closely linked genetic markers were detected by gwm212 and gwm292 that were also used in this current research.

In this study, it has been shown that gwm292 has a high specificity (90 %) for *Vrn-D1* gene and therefore can be used as a diagnostic tool in breeding programs. However considering that this gene plays an important role not only for inducing earliness in flowering but also for stress adaptation during grain filling stage, more research to find molecular markers linked to *Vrn-D1* gene is encouraged to be done. For this purpose it is worth to employ AFLP technique that uses large numbers of primer combinations by combining different restriction enzyme systems or modifying the number of selective nucleotides included in the main amplification step. This will increase the chance to yield more markers linked to this gene, so the markers found can be later applied for breeding of *Vrn-D1* gene to accompany another existing markers for *Vrn-A1* and *Vrn-B1* genes.

4.3 Genetics of photoperiod genes

Martinic (1975) emphasized that a low response to photoperiod, as assessed in terms of days to heading, appears to be a standard characteristic of many of the new, high yielding and widely adapted varieties of both spring and winter wheat. He further stressed that low sensitivity to photoperiod seems to be of special importance in latitudes about and below 45°. Law et al. (1978) added that these day length insensitive wheats are essential to the agriculture of many countries where more than one harvest a year is required or high summer temperatures restrict the growing of wheat to the winter months.

Photoperiod insensitivity is controlled primarily by a homeologous series of genes *Ppd-D1*, *Ppd-B1*, and *Ppd-A1* located on chromosome 2D, 2B and 2A respectively (Welsh et al., 1973; Law et al., 1978). Of these genes, *Ppd-D1* is considered the most potent (Law et al., 1978) and is probably the gene most likely to occur in European varieties (Scarth and Law, 1983), hence has been more intensively studied in the past in comparison with other two major *Ppd* genes. There is, however, a wide area across the middle of Europe, including Germany and UK, where variable climatic conditions can favour *Ppd-D1* one year and *ppd-D1* the next. It is likely that across this intermediate region there may be adaptive advantages for *Ppd-B1* or *Ppd-A1* that are said to be less potent than *Ppd-D1* (Worland et al., 1998). Therefore, many studies are conducted in recent years to prove this theory.

Two of three major photoperiod insensitive genes, i.e. *Ppd-A1* and *Ppd-B1* have been planned to be under study in this work. For this purpose, three populations consisting of one population segregating for *Ppd-A1* and 2 populations for *Ppd-B1* have been employed.

However, as confirmed in previous chapter it is known that DH population from the cross of Mercia x Mercia(C5912A) segregates for *Ppd-B1* instead of *Ppd-A1*. It is not clearly understood why this population does not segregate for *Ppd-A1* gene. It seems that C591 and Mercia are not the parents of this population. This hypothesis is supported by the absence of polymorphism from nine microsatellite markers specific for chromosome 2A in parental lines. However, it is interesting to note that both of the pools and offspring of this population could be differentiated by 1 SSR (*Xgwm257*-2BS) and several AFLP markers from chromosome 2B, while the same markers were monomorphic in parental lines. This fact explains the inappropriate information between parental lines and offspring, leading to a conclusion that the identity of parental lines is not correct and the gene segregating in this population is *Ppd-B1*. Since the real parents are not known, the name of this population is still used for the next discussion.

The parental lines of another two populations employed for identifying markers associated with *Ppd-B1* gene in this study are current breeding materials often used for photoperiod study or breeding. Recent survey of photoperiodic genes in European wheats conducted by Worland et al. (1994) located Mercia from the UK, Arche and Tremie from France, Ibis, Ramiro and Ronos from Germany and the older Russian variety Mironovskaya 808 in a small group of varieties with an intermediate sensitivity to photoperiod flowered with a regular 50 to 100 days delay. It was proposed that this group of varieties might carry a different photoperiod sensitive gene or allele to other European varieties. Similarly, Marquis has been known to carry photoperiod sensitive gene in which substitution line of CS(Marquis 2B) proved to be much more photoperiod sensitive than CS (Scarth and law, 1983). CS it self is a spring wheat that will flower without vernalization, although it is slightly responsive. It is also known that besides carrying *Vrn-D1* allele (McIntosh et al., 1998; Law, 1966), CS also carries *Ppd-B1* allele (McIntosh et al., 1998; Scarth and Law, 1983). Therefore, the use of CS either as female parent or as a donor in CS x CS(Marquis2B) population and Mercia x Mercia(CS2B) population, respectively gives the certainty that both of populations carry *Ppd-B1* gene. Common markers found among three populations also support the fact that Mercia x Mercia(C5912A) population really segregates for *Ppd-B1* gene. The use of three populations carrying the same gene enabled to learn in detail segregation pattern, population type, genetic and physical mapping, and also behaviour of the markers, so the drawing of the conclusion from this study is reliable.

4.3.1 Segregation distortion

Skewed segregation ratios were observed in DH populations Mercia x Mercia(C5912A) and Mercia x Mercia(CS2B). In Mercia x Mercia(C5912A) population, all marker loci deviated slightly from the expected 1:1 ratio, while in Mercia x Mercia(CS2B) population segregation distortion was found for 7 of 20 (35 %) marker loci. All of these distorted markers skewed towards the Mercia genotype. Interestingly, all markers in CS x CS(Marquis2B) population segregated normally following a 1:1 segregation. This clearly indicates that segregation distortion does not depend on the type of markers (AFLP, SSR and CAPS).

Theoretically, all population types may show distorted segregation. However, it seems that there is a correlation between frequency level of distorted markers and population type. In rice, Xu et al. (1997) found that RI populations had significantly higher frequencies of distorted markers than doubled-haploid, backcross and F_2 populations. Furthermore, they also found chromosomal regions associated with marker-segregation distortion in six segregating populations. If a gene that causes distorted segregation is segregating in a population then markers close to it would tend to exhibit distorted ratios (Zamir and Tadmor, 1986). And if several populations are segregating for the same gametophyte factors or other unknown genes that cause segregation distortion, then these populations will exhibit distorted segregation at the same chromosomal regions. This was not observed for two DH populations used, even the locations of distorted loci were completely different, explaining that distorted segregation in these two populations happened randomly. The slight deviation shown by distorted loci in this study seems more to be caused by the moderate size of population. This means that the number of loci showing skewed segregation could be reduced if larger population sizes would be used.

High levels of segregation distortion have already and frequently reported for other plants. The percentage of loci showing segregation distortions was highly variable: 30 % in banana (Faure et al. 1993), 25.5 % in potato (Gebhardt et al. 1989), 12-59 % in rape (Kianian and Quiros, 1992), 65 % in maize (Wendel et al. 1987) and 68 % in tomato (Paterson et al. 1988).

A variety of physiological and genetic factors could cause segregation distortion (Grant, 1975). Mechanisms for preferential segregation include pollen-tube competition (Mangelsdorf and Jones, 1926; Levin and Berube, 1972; Liedl and Anderson, 1993), pollen lethals (Rick, 1966), preferential fertilization (Schwemmle, 1968; Gadish and Zamir, 1986) and selective elimination of zygotes (Rick, 1963). In the present experiment, segregation distortion was

just observed in the female and absent in the male parent, suggesting the occurrence of some form of selection at the female gamete level. Distortion segregations have indeed been previously described as a consequence of genetic divergence between the parent plants (Kianian and Quiros, 1992).

4.3.2 Development of CAPS marker for *Ppd-B1* gene

The screening or large populations in breeding programmes would benefit from the development of simple, PCR-based molecular marker assays (Parker and Langridge, 2000). In recent years, successful conversion of AFLP into STS has been reported in several plant species including potato (Brigneti et al., 1997), barley (Decousset et al.), carrot (Bradeen and Simon, 1998), soybean (Meksem et al., 2001), asparagus (Reamon-Büttner and Jung, 2000) and pea (Stackelberg et al., 2003). In wheat, most of recent STS were also developed from AFLP (Quo et al., 1999; Shan et al., 1999; Prins et al., 2001, Mohler et al., 2002, Parker and Landridge, 2000; Guo et al., 2003). AFLP-derived STS can be used to create isogenic lines for fine structure mapping of QTLs or to introgress genes into adapted cultivars (Tanksley and Nelson, 1996; Tanksley et al., 1996). Reliable, codominant and cost effective markers are necessary for high throughput genotyping tasks (Bradeen and Simon, 1998).

In this study, two AFLP bands from the same AFLP PC (S20M13) were assayed and one was successfully converted into STS. Sequence analysis of early and late-flowering progeny (of *Ppd-A1*) amplified by STS *Ppd515* revealed that the two alleles differed in size by 20-bp insertion/deletions as well as 4 single nucleotide polymorphism. A combination of point mutations and small indels observed between the two alleles identified in this study was in good accordance with the result observed in another cases where allelic sequences derived from a converted AFLP have been compared (Bradeen and Simon, 1998; Paltridge et al., 1998; Parker and Langridge, 2000). By employing *Hin6I*, a 4-bp cutter restriction enzyme, a clear distinction between early and late-flowering progeny could be shown. A single band in the size of 355-bp was obtained from late flowering–progenies amplified by this CAPS marker, which was later designated as whs2002. Further mapping across this population revealed that this CAPS marker co-segregated with the original AFLP marker (S20M13-515), confirming that the correct sequences were amplified.

As described in previous chapter, the similar genotyping pattern of CIMMYT lines shown by this CAPS marker with gwm257 which is specific to chromosome 2B (Röder et al., 1998b) and the absence of amplification of this marker in N2B resulted in the drawing of conclusion that this CAPS marker is linked to wheat chromosome 2B. However, the

contradictory linkage phase of this marker to Mercia x Mercia(C5912A) and CS x CS(Marquis2B) population was observed. This marker was linked to Mercia allele in Mercia x Mercia(C5912A) population and linked to CS allele in CS x CS(Marquis 2B) population possibly explaining the absence of polymorphism in the third population Mercia x Mercia(CS2B), in which this CAPS marker exhibited the same marker alleles in both parents. Although this marker is linked to *Ppd-B1*, it can be concluded that it is not a diagnostic marker for this gene. However, this does not mean that this marker can not be used at all. By screening the parents and bulk segregants from populations segregating for *Ppd-B1* gene first, it can be easily found out in which linkage phase this CAPS marker is linked to *Ppd-B1* gene. The obtained information could then be used as an indicator for genotyping the offspring of the population.

Several difficulties have been reported for the conversion of AFLPs into STS markers. These problems included a target polymorphic band may contain contaminating fragments from adjacent bands (Prins et al., 2001), the size of AFLP fragment is too short for designing appropiate PCR primers (Bradeen and Simon, 1998) and the loss of the original polymorphism after specification process leading to a poor conversion success rate (Shan et al., 1999; Stackelberg et al., 2003). In this study, the loss of the original polymorphism was also faced by STS *Ppd-272*. There was no difference found from sequence analysis of early and late-flowering progeny amplified by this STS marker. It seems that the polymorphism shown by the original AFLP band was due to point mutations that were located at or within the restriction sites of AFLP primer or at the site where the selective nucleotides matched the fragment.

Von Stackelberg et al. (2003) could only convert 3 of 12 analysed AFLPs into STS markers. A poor conversion success rate of just 3 out of 16 led Shan et al. (1999) to conclude that STS derivation from AFLP is an inefficient process. However, under current conditions, since AFLP offers the best available means of uncovering linked DNA sequences, efforts to improve its convertibility will be worthwhile (Prins et al., 2001).

To solve their problem, Von Stackelberg et al. (2003) developed a simplified procedure to convert AFLP bands into single PCR products. They mentioned that if the direct way to convert an AFLP into a STS marker failed, they used primers corresponding to the original AFLP selective primers with an additional two up to five selective bases from the sequence clone. The theoretical concept behind this is each additional selective base will reduce the group of amplified fragments on average by 75 %. If in total six selective bases (three on each original AFLP selective primer) lead to a maximum of 150 amplified bands just four

additional selective bases more should reduce the number of amplified bands to a single band. The original polymorphism which is captured in the pre-amplification template can be displayed as a single locus. With this technique which is named single-loci sequence-specified AFLP (ssAFLP), five additional AFLPs could be converted and analysed as single bands in agarose gel. Since AFLP marker S20M13-272 has been shown to be tightly linked to *Ppd-B1* in three different populations, it is worth to try this method and convert the AFLP marker into an ssAFLP. The primers obtained with this technique, if successful, would be very useful as a simple tool for tagging *Ppd-B1* gene.

4.3.3 Linkage mapping

The genetic mapping of three precise populations in this study supports the evidence that wheat chromosome 2B carries photoperiod insensitive gene *Ppd-B1* as proposed earlier by Scarth and Law (1983). The combination of PCR based marker techniques, such as AFLP and SSR, with bulked segregant analysis proved to be an efficient way of identifying markers closely linked to the trait of interest.

Although in general the marker order in three populations is conserved, the markers were evenly distributed only in the linkage group for population III. Two gaps in the size of 17.3 and 10.5 cM, respectively were observed in population II, while in population I linked markers were detected only on the distal side of *Ppd-B1* locus. Three possibilities have been proposed for this condition by Barett et al. (2002) including a very close donor-recipient crossover event in the parent backgrounds, a low number of markers screened, or to low polymorphism levels proximal to the locus. Since more than 300 markers have been employed for screening the DNA bulks of this population, the second possibility can be excluded.

The use of three different populations carrying the same gene in this study enabled to answer the crucial question in MAS whether the markers found in a certain cross can also be applied to different crosses. It is clear observed that not all of markers were transferable among populations. By choosing the population whose amount of markers between two populations is smaller as a base for comparison, the amount of common markers between two populations can be described as following: 67 % (4 of 6 markers) between population I and II, 50 % (3 of 6 markers) between population I and III and 75 % (8 of 12 markers) between population II and III. This value decreases significantly to be 33 % (2 of 6 markers) when all three populations are compared among each other. This result demonstrates how difficult it is

to find common markers for a target gene, which can be used universally in different populations.

Apart from that information, it can be inferred that the behaviour of markers in different populations can be as following: (1) specific markers which are linked to the gene of interest in just one cross and can not be applied to other crosses, (2) common markers which always amplify the same locus in different crosses. These informations are very important for the breeders before they begin to apply MAS as a tool for breeding. The first marker type should just be strictly used for breeders who really work with the same population as published for these markers. Otherwise, it is recommended to use common markers that have been validated, especially if no information is available for the parental lines.

Since several genetic maps around *Ppd-B1* using RFLP markers have been established, it would be interesting to test all obtained markers from this study to another existing *Ppd-B1* mapping populations, and vice versa. From this kind of validation, it could be obtained more true common markers for *Ppd-B1* gene. Furthermore, the integration of different molecular markers would result in the fine mapping around *Ppd-B1* region.

4.3.4 Physical mapping of chromosome 2BS of wheat

Methods for constructing physical maps can be classified into two basic categories: molecularly based and cytogenetically based. Molecularly based methods include the construction of contigs using libraries of genomic fragments, and long range restriction mapping using rare cutting enzymes (Cheung et al., 1991). These methods are useful for fine-structure mapping of small areas of the genome, but are not feasible for physical mapping of the entire wheat genome. Cytogenetically based methods include in situ hybridisation (Jiang and Gill, 1994), genetic mapping of polymorphic chromosome markers such as C-bands (Linde-Laursen, 1979), and deletion mapping (Werner et al., 1992). Among these methods, deletion mapping is often employed for physical mapping since this technique is efficient in resolving proximal clusters of molecular markers (Werner et al., 1992), eliminating the requirement for intragenomic poymorphism and can be used to localize agronomically important genes to relatively small chromosomal regions (Faris et al., 2000).

The physical map of chromosome 2BS of wheat constructed using deletion lines in this study is in a good agreement with genetic map constructed with BSA method, in which all 9 markers were distributed just in the distal one-half of the arm. According to Michelmore et al. (1991), a genetic window of 25 cM at both sides of the target window is opened and the size of the window that can be detected depends on recombination during population

development. The absence of recombination in the proximal one-half of the arm supported this theory and also indicating that the genetic map length in this study is derived from recombination events in the distal 50 % of 2BS chromosome arm. The markers order on physical map indicating that the location of *Ppd-B1* locus should be on the terminal part of the distal region. This observation is in agreement with an earlier study of Lukaszewski and Curtis (1993) who reported that in the B-genome chromosome of wheat, physical recombinations were concentrated in the distal chromosome segments and to be infrequent or absent in proximal segments. As a result, distal chromosome regions appear extended, and proximal segments compressed, in genetic maps of wheat (Jampates and Dvorak, 1986; Curtis and Lukaszewski, 1991; Werner et al., 1992; Gill et al., 1993, 1996 a, b; Kota et al., 1993).

The physical maps constructed using deletion lines indicated that certain regions of the chromosomes exhibit a high gene density (Gill et al., 1993, 1996 a, b; Hohmann et al., 1994; Delaney et al., 1995; Mickelson-Young et al., 1995; Weng et al., 2000), and when compared to recombination–based maps, the gene rich regions preferentially participated in recombination (Gill et al., 1996 a, b). In this study, it was also observed that the markers did not distribute equally in the first four bins of distal region but concentrated in two deletion intervals. No markers were mapped between fraction length of 0.75 and 0.83. This result is in good accordance with previous physical mapping of the group-2 chromosomes of wheat conducted by Delaney et al. (1995) and Röder et al. (1998a) who could not assign a marker in the deletion interval between fraction length of 0.75-0.84 and 0.73-0.83, respectively. From these results, it appears that fraction length 0.73-0.83 does not contribute to the recombination of distal 2BS chromosome region. The current physical map also confirmed the location of microsatellite loci *Xgwm257* and *Xgwm148* as previously mapped by Röder et al. (1998a). Both of these markers in this study were mapped exactly at the same deletion intervals like before, showing the stability of deletion lines and the high reproducibility of the microsatellite markers used.

In wheat as well as in other cereals, it has been demonstrated that linkage mapping is not an efficient method for determining the physical location of genes, due to variation in recombination along the length of a chromosome (Snape et al., 1985; Werner et al., 1992; Gill et al., 1996a, b). Neu et al. (2002) added that close genetic linkage can be due to either close physical linkage of loci or suppressed recombination. Therefore, comparison of genetic map and physical map in this study is very useful to know the real situation. The cluster of three markers in the first deletion interval revealed that the close genetic linkage of all loci distal to *Ppd-B1* locus is due to close physical linkage of loci. The same reason can also be addressed

to explain close genetic linkage of 6 loci that were mapped in the largest deletion interval between deletion lines 2BS-3 and 2BS-6. However, suppressed recombination seems to be responsible for the close genetic linkage between *XE38M53-146* and *Ppd-B1* locus in population III since it has been shown that the location of *Ppd-B1* locus on the genetic maps of all three populations is near to the distal markers that have been located in the same deletion interval, while marker *XE38M53-146* is separated by two deletion intervals to the first deletion interval. This hypothesis is corroborated by genetic mapping in population II where a 7-fold increase of recombination frequency in the *Ppd-B1 – XE38M53-146* interval (25.7 cM) was observed compared with population III (3.6 cM). This hypothesis will be clarified later by further physical mapping of *Ppd-B1* gene.

Deletion mapping, like aneuploid mapping, requires intergenomic polymorphism among orthologous loci while linkage mapping required polymorphism at individual loci among the parents of a segregation population. Consequently, four AFLP loci that could not be mapped in this study (due to the lack of intergenomic polymorphism) have been mapped in segregating populations and, conversely, several loci mapped in this study could not always be mapped in three segregating populations due to the lack of polymorphism. A combination of the two methods allows mapping a greater number of loci. Segregating populations are easier to produce than deletion lines but the latter have the advantage that physical positions of loci can be unambiguously determined (Weng et al., 2000).

4.4 Perspective and application of MAS

The regulation of heading time is one of the major objectives in wheat breeding because it is essential that the wheat plants flower at times appropriate to particular environmental conditions. This attempt is very important to gain maximum yield potential and to avoid environmental stress like frost in the winter or drought and high temperatures in the summer. Therefore, the markers gwm292 for *Vrn-D1* and gwm257 and whs2002 for *Ppd-B1* identified in this study are important from an economic and breeding perspective. In addition, the populations used were generated using current breeding material, so that the results obtained are applicable and relevant to wheat breeding in general. The available of linked markers in different population backgrounds, either from this study or from earlier studies, provides several choices for the breeder to use right and suitable markers for certain cross they are working with. Using linked markers as tags, breeders will be able to begin selecting for genotypes carrying *Ppd-B1* and *Vrn-D1* genes earlier in breeding program.

One of the important requirements for the effective implementation of molecular markers is the use of friendly markers. Markers need to be cheap and easy to use. The most and widely used marker system and have been the basis for most work in wheat is based on RFLPs (Langridge and Chalmers, 1998). However, RFLP analysis has some limitations, since it is time consuming and labour intensive. Further, because of the low frequency of RFLPs in wheat, this approach has been relatively less useful in this crop. This low frequency is sometimes attributed to the polyploid nature, high proportion of repetitive DNA, large genome size and recent origin of wheat (Gupta et al., 1999). Therefore, emphasis is now shifting to PCR-based assay systems, which are expected to be cheaper and more easily used than RFLPs. The results of this study demonstrated that both of SSRs and AFLPs combined with bulked segregant analysis were easy to be used and very useful in identifying markers linked to genes, providing a basis for the development of alternative plant breeding strategies.

Another important application of MAS is for pyramiding the genes. It has been reported that each *Ppd/Vrn* gene has good adaptability for certain environment. In order to increase the adaptability and yield potential, multiple *Ppd/Vrn* genes could be pyramided in newly released varieties. Based on conventional screening techniques, it is difficult to determine whether individual lines carry more than one *Ppd/Vrn* gene when genes from both parents are combined in the progeny. Successful pyramiding of different resistance genes using MAS has been reported by Toojinda et al. (1998) who were able to rapidly develop barley germplasm carrying multiple stripe-rust resistance alleles. The *Xa21* gene (resistance to bacterial blight), the *Bt* fusion gene (for insect resistance) and the chitinase gene (for tolerance of sheath blight) were combined in a single rice line by Datta et al. (2002). In wheat, three powdery mildew resistance gene combinations, *Pm2* + *Pm4a*, *Pm2* + *Pm21*, *Pm4a* + *Pm21* were also successfully integrated into an elite wheat cultivar 'Yang158' by Liu et al. (2000). Also pyramiding of monogenically controlled resistance has been adopted by breeding industry (Wenzel et al., 2001). The same approach could be also used for genes responsible for adaptation. The availability of tightly linked markers for these photoperiod and vernalization genes will allow plants carrying more than one gene to be identified in breeding programs.

Apart from containing *Ppd-B1* gene, wheat chromosome 2BS has been reported to carry several important agronomic genes such as yellow rust (*Yr27* and *YrCV*), plant height, grain weight/ear (Börner et al., 2002), leaf rust (*Lr23*; Nelson et al., 1997), stem rust (Sr36; Knott, 1989), and earliness per se genes (Shindo et al. 2003). Therefore the informations obtained from this current research can be applied not only for breeding of *Ppd-B1* gene, but also for another genes located on 2BS chromosome as mentioned above. Furthermore, the

information from physical location of markers mapped in this study will also enrich the existing physical mapping of RFLP and SSR marker that previously located on 2BS chromosome.

Current assays for selecting different photoperiod and vernalization responses in the field are time consuming, labour intensive and therefore expensive to perform. Many of these assays are only used to test mid to late generation material that has already passed through several seasons of field trials. The ability to screen lines for these traits early in the program using molecular genotyping will allow undesired material to be discarded. Furthermore, prevailing season, which is one of the barriers in current breeding can be eliminated. Molecular markers are not environmentally regulated. Thus, with MAS it is now possible for the breeder to conduct many rounds of selection in a year. The use of rapid DNA extraction procedures along with PCR based markers such as STSs, SSRs, or ssAFLPs makes MAS even more attractive. However, molecular markers are not expected to replace phenotypic selection in breeding programs. Rather, they should be integrated as an additional selection tool for breeders, so that the efficiency and accuracy of selection can be significantly improved. The current technology employing expression profiling of precise genetic stock using cDNA array enables to find genes involved in photoperiod and vernalization response. This approach is worth to be tried for cloning *Ppd*/*Vrn* genes, so that perfect markers for MAS can be developed.

5 Summary

The work presented in this thesis was conducted with the broad aim of identifying molecular markers linked to major genes associated with photoperiod (*Ppd*) as well as vernalization (*Vrn*) response in hexaploid wheat. The approach used combined bulked segregant analysis (BSA) method with 2 PCR based marker techniques (SSR and AFLP).

A single chromosome recombinant line (SCRL) population derived from a cross between Chinese Spring (CS) and CS(Hope5D) was employed to study *Vrn-D1* gene. Previous screening of 150 AFLP and 2 SSR markers specific for wheat chromosome 5D, gwm212 and gwm292, between the parental lines and DNA bulks revealed that only gwm292 was polymorphic. Genotyping of 94 individuals showed that gwm292 was closely linked to the *Vrn-D1* gene, with a map distance of 2.81 cM. However, validation of this marker using 52 CIMMYT wheat cultivars and 8 tester lines (Triple Dirk series) revealed that the allele size of CS (221 bp) was not an exclusive informative marker for *Vrn-D1* since Triple Dirk E, which is proved to carry also *Vrn-D1* showed a marker allele of 215 bp. Further comparison between allele data from gwm292 and phenotypic data in a set of 36 CIMMYT wheat cultivars showed that there was a high correlation between 215-bp marker allele from gwm292 and cultivars carrying *Vrn-D1* gene with just four misclassifications from 36 lines, showing the potential of this marker to be used as a diagnostic tool in breeding programs.

Photoperiod insensitive gene *Ppd-B1* was investigated using three segregating populations. Four SSR primer pairs from the short arm of chromosome 2BS, 75 *Sse*8387I + NN/*Mse*I + NN and 183 *Eco*RI + ANN/*Mse*I + CNN primer combinations were employed to screen the parents and the DNA bulks assembled from mapping populations CS x CS(Marquis2B) and Mercia x Mercia(CS2B). Two microsatellite markers and two AFLP markers were polymorphic in CS x CS(Marquis2B), while one microsatellite marker and 18 AFLP markers were polymorphic in Mercia x Mercia(CS2B) population. However, marker density could be increased by exchanging common markers to 20 in Mercia x Mercia(CS2B) and 11 in CS x CS(Marquis2B).

Based on marker data analysis, a third population, Mercia x Mercia(C5912A) which was previously presumed to segregate for *Ppd-A1*, was found to segregate for *Ppd-B1* gene. A total of 331 AFLP markers and nine microsatellite primer pairs specific for chromosome 2A were used to survey the DNA bulks and two parents of Mercia x Mercia(C5912A) population for polymorphism. Three markers (E37M47-319, E39M60-403 and S20M13-272) co-

segregated and were tightly linked to *Ppd-B1* gene, with a map distance of 1.4 cM, while marker S20M13-515 co-segregated with the *Ppd-B1* gene.

Two markers S20M13-272 and S20M13-515 were converted into simple PCR markers. Sequence analysis from early and late-flowering individuals amplified by these two STS markers revealed no sequence alterations for the 272-bp AFLP-derived STS markers, whereas for the 515-bp AFLP-derived STS markers four SNPs and a 20-bp indel could be detected. The indel site harbours a recognition site at position 355-bp for the *Hin*6I restriction enzyme. Using this sequence information, a cleaved amplified polymorphic sequence (CAPS) marker has been developed and designated as whs2002. This marker showed an additional band in the size of 355 bp from late flowering progeny, offering a simple method for tagging *Ppd-B1* gene.

The use of three populations carrying the same gene enabled to answer the crucial question in MAS whether the markers found in certain cross can also be applied in different crosses. Number of common markers between two populations varied between 50 to 75% and decreased significantly to be 33% when all three populations were compared among each other, indicating the difficulty to find friendly markers for a target gene which can be used universally in different genetic backgrounds. The physical map of chromosome 2BS of wheat constructed using deletion lines is in a good agreement with the genetic map constructed with BSA method, in which all 9 markers were distributed just in the distal one-half of the chromosome arm. Finally, the genetic and physical maps were compared to determine whether close genetic linkage of the markers is caused by close physical linkage of loci or suppressed recombination. This information is very important and should be known before one decides to apply markers for breeding purpose. Only common markers whose close genetic linkage to the target gene are due to close physical linkage of loci can be reliably used and worth to be converted into simple markers, such as STS or ssAFLP.

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7 Appendices

7.1 Chemicals

Substance	Supplier	Specification
Agarose Seakem	FMC, Rockland	
Agarose Seaplaque	FMC, Rockland	
Ammoniumpersulfat	Amresco, Ohio	>98%
Ampicillin	Boehringer, Mannheim	pure
ATP	New England Biolabs, Beverly	
Bacto-agar	Difco, Detroit	
Bacto-typton	Difco, Detroit	
Boric acid	Amresco, Ohio	pure
Bromophenolblue	Riedel-de-Haen, Seelze	p.a.
Chloroform	Roth, Karlsruhe	p.a.
CTAB	Sigma, St. Louis	p.a.
Dextran blue	Merck, Darmstadt	
DNTP-Mix	Pharmacia, Uppsala	
EDTA	Serva, Heidelberg	p.a.
Ethidiumbromid	Serva, Heidelberg	pure
Formamide	Merck, Darmstadt	98 %
Genescan 500 ROX	Perkin Elmer	
Genescan 500 TAMRA	Perkin Elmer	
Glycerin	Roth, Karlsruhe	99.5 %
HCl	Baker, Phillipsburg	p.a.
Isoamylalkohol	Baker, Phillipsburg	p.a.
Isopropanol	Roth, Karlsruhe	p.a.
Long ranger gel-solution	FMC, Rockland	
B-Mercaptoethanol	Merck, Darmstadt	p.a.
$MgCl_2$	Merck, Darmstadt	p.a.
NaAc	Merck, Darmstadt	p.a., cryst.
NaCl	Roth, Karlsruhe	p.a.

Substance	Supplier	Specification
NaOH	Merck, Darmstadt	p.a.
NH_4Ac	Riedel-de-Haen, Seelze	p.a.
Rnase	Roth, Karlsruhe	
TEMED	Amresco, Ohio	p.a.
Tris	Riedel-de-Haen, Seelze	p.a.
Urea	Amresco, Ohio	p.a.

7.2 Abbreviations

°C	degrees celcius
µg	microgram
µl	microlitre
µM	micromolar
A	Adenine
AFLP	amplified fragment length polymorphism
APS	ammonium per sulfate
bp	base pair
BSA	bulked segregant analysis
C	Cytosine
CAPS	cleaved amplified polymorphic sequence
chr.	chromosome
cM	centimorgan
cryst	crystalline
CS	Chinese Spring
CTAB	cetyltrimethylammonium bromide
dCAPS	derived cleaved amplified polymorphic sequence
DNA	deoxyribonucleic acid
DNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
FL	fraction length
G	Guanine
LOD	logarithm of odds
MAS	marker assisted selection

ml	millilitre
mM	millimolar
ng	nanogram
NIL	near isogenic line
p.a.	pro analysis
PCR	polymerase chain reaction
PCs	primer combinations
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
Rnase	ribonuclease
SCAR	sequence characterized amplified regions
SCDH	single chromosome doubled haploid
SCRL	single chromosome recombinant line
SNP	single nucleotide polymorphism
ssAFLPs	sequence-specified amplified fragment length polymorphisms
SSM	slip strand mispairing
SSR	simple sequence repeat
STS	sequence tagged site
T	Thimin
TD	triple dirk
v/v	volume/volume
w/v	weight/volume

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