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K. Iwaki · J. Nishida · T. Yanagisawa · H. Yoshida
K. Kato

Genetic analysis of *Vrn-B1* for vernalization requirement by using linked dCAPS markers in bread wheat (*Triticum aestivum* L.)

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Abstract To identify a molecular marker closely linked to *Vrn-B1*, the *Vrn-1* ortholog on chromosome 5B, sequence polymorphism at four orthologous RFLP loci closely linked to the *Vrn-1* gene family was analyzed by using near-isogenic lines of "Triple Dirk." At *Xwg644*, a RFLP locus, three types of nucleotide sequence differing by the number of (TG) repeats, two or three times, and base changes were detected. A $(TG)_3$-type sequence proved to be specific to chromosome 5B by nulli-tetrasomic analysis, and substitution of single nucleotide (C/T) was detected between TD(B) carrying the former *Vrn2* allele and TD(C) carrying the *vrn2* allele. A mismatch primer was designed for dCAPS analysis of this single nucleotide polymorphism (SNP). Polymorphism was successfully detected between two NILs, through nested PCR by using a $(TG)_3$-specific primer (1st) and a dCAPS primer (2nd) followed by a *Nsi*I digest. The analysis of a BF_2 population [(TD(B)//TD(C)] revealed the close linkage (1.7 cM) between WG644–5B and *Vrn2*. It was therefore concluded that the former *Vrn2* locus is located on chromosome 5B and equivalent to *Vrn-B1*.

Keywords dCAPS · Orthologous gene · Synteny · Vernalization requirement · Wheat

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K. Iwaki · J. Nishida · K. Kato (✉)
Faculty of Agriculture, Okayama University,
1-1-1 Tsushima-Naka, Okayama 700-8530, Japan
e-mail: kenkato@cc.okayama-u.ac.jp, Fax. +81–86-251-8388

T. Yanagisawa · H. Yoshida
National Agriculture Research Center,
3–1-1 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

Present address:
K. Iwaki, Ohmi Research Center, Flower Division,
Suntory Limited, Youkaichi 523-0063, Japan

Introduction

The heading time of wheat is a complex character determined by three traits, i.e. earliness *per se*, photoperiodic response and vernalization requirement (Yasuda and Shimoyama 1965; Hoogendoorn 1985a, b; Kato and Yamagata 1988). Among these traits, vernalization requirement refers to the requirement of low temperature necessary for the induction of reproductive growth, and it is generally accepted that this trait is an important character for adaptation to cold winters. Vernalization requirement is controlled by five major genes, *Vrn-A1*, *Vrn2*, *Vrn-D1*, *Vrn4* and *Vrn-B4* as reviewed by Flood and Hallolan (1986), Worland et al. (1987) and McIntosh et al. (1998). However, it is very difficult to identify the *Vrn* genotype of wheat plants by autumn-sowing in the field, since wheat is fully vernalized during cold winters (Gotoh 1975; Yasuda 1984; Kirby et al. 1985). For the selection of adaptive *Vrn* genotypes in breeding programmes, molecular markers for each *Vrn* gene should be developed.

Intensive chromosome mapping has been carried out mainly by using RFLPs, and several RFLP loci linked to *Vrn* genes have been reported; *Xcdo504*, *Xwg644* and *Xpsr426* linked to *Vrn-A1* (Galiba et al. 1995), *Xrz395* and *Xbcd450* linked to *Vrn-A1* and *Vrn-D1* (Nelson et al. 1995; Kato et al. 1999). However, RFLPs often show little or no polymorphism among improved cultivars (Bryan et al. 1999). For the other genes, although *Vrn2* is important for the adaptation of spring-type wheats in the southern part of Europe, RFLP markers linked to *Vrn2* have not been identified. 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 1985a; Iwaki et al. 2001b), and *Vrn4* on chromosome 5D (Kato et al. 1993). Reflecting such a confusion, McIntosh et al. (1998) summarized that the genes formerly designated as *Vrn4* and *Vrn2* are probably the same, or allelic, and that the two genes are designated as *Vrn-B1* in the new nomenclature system. However, inde-

pendence of *Vrn2* and *Vrn4* was clearly shown by test crosses between four near-isogenic lines (NILs) (Gotoh 1979; Kato, unpublished), and by the existence of cultivars carrying either *Vrn2* or *Vrn4* (Iwaki et al. 2001a). Since vernalization requirement is a quantitative trait, molecular-based approaches should be introduced to solve such a confusion.

Polymorphism among wheat cultivars can be relatively easily detected by PCR-based methods such as microsatellites (Röder et al. 1995) and amplified fragment length polymorphism (AFLP, Vos et al. 1995). AFLP is beneficial for the development of molecular markers even for target genes which are not localized on a chromosome map. AFLP markers for *Pm1c*, *Pm4a* (Hartl et al. *1*999), *Pm24* (Huang et al. 2000) and flour color (Parker et al. 1998) have been developed in wheat. On the other hand, in the case of mapped genes, cleaved amplified polymorphic sequences (CAPS, Talbert et al. 1994; Komatsuda et al. 1998; Hernandez et al. 1999) and DNA sequence polymorphism (Bryan et al. 1999) at a linked RFLP locus can be used to develop molecular markers. Single nucleotide polymorphisms (SNP) among RFLP locus-specific PCR products can be converted to PCR-based marker such as CAPS and derived CAPS (dCAPS, Michaels and Amasino 1998; Neff et al. 1998) markers.

The comparison of RFLP maps among several crop species of the *Triticeae* has indicated conserved synteny (Devos and Gale 1997); and *Vrn-A1*, *Vrn-B1*, *Vrn-D1* in common wheat, *Vrn-A^{m}1* in *Triticum monococcum*, *Vrn-H1* in barley and *Vrn-R1* in rye have proven to be members of an orthologous gene set for *Vrn-1* (Galiba et al. 1995; Dubcovsky et al. 1998). Taking the synteny between homoeologous group-5 chromosomes into consideration, an orthologous DNA sequence corresponding to the RFLP locus linked to one of the *Vrn-1* orthologs should also link to each member of the series. In the present study, therefore, sequence polymorphism at four orthologous RFLP loci was analyzed using NILs of "Triple Dirk", carrying *Vrn2* or *Vrn4*, to develop PCR-based markers closely linked to *Vrn-B1*.

Materials and methods

Plant materials

Spring type NILs of a wheat cultivar "Triple Dirk", TD(B) and TD(F), and a winter type NIL [TD(C)] were examined in the present study. These NILs were developed by Pugsley (1971, 1972), and the former two NILs carry *Vrn2* and *Vrn4*, respectively. TD(B) and TD(F) have been further backcrossed twice and four-times with TD(C), and their selfed BF_2 populations were prepared as hybrid populations segregating for *Vrn2* and *Vrn4*, respectively. The other NILs of "Triple Dirk", TD(D) and TD(E) which carry *Vrn-A1* and *Vrn-D1* respectively, were also examined, as well as the original cultivar (TD) carrying both *Vrn-A1* and *Vrn2*. Nulli-tetrasomic lines of "Chinese Spring" were used for chromosomal assignment of each PCR product.

Extraction of total DNA

Ethiolated seedlings of the NILs, grown at 20°C under dark conditions for ten days, were separately ground in liquid nitrogen, and total DNA was extracted following the procedure of Murray and Thompson (1980) with minor modifications. As for the BF_2 populations, total DNA was extracted from a young green leaf (0.2–0.3 g) of each plant at 1–3-months old.

Primer design and PCR amplification

Among RFLP loci linked to *Vrn-1* on the long arm of homoeologous group-5 chromosomes, four loci, *Xpsr145* (Laurie et al. 1995), *Xpsr426*, *Xbcd450* and *Xwg644*, were investigated in the present study. Four RFLP probes were ligated into the pGEM-T Easy Vector (Promega), and nucleotide sequence was determined by using the Dye-Primer method (Smith et al. 1986) with a DSQ-1000 DNA sequencer (Shimadzu, Japan). Based on their nucleotide sequence, specific primer pairs (20–21 mer) were designed to amplify the respective region corresponding to each RFLP probe (Table 1). PCR amplification was performed in a volume of 10 µl, containing 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.25 units of *Taq* polymerase (Pharmacia), 0.1 mM of dNTP, 0.2 µM of Primer, and 25 ng of template DNA, by using the Program Temp Control System PC-700 (ASTEC, Japan). After an initial denaturation step at 95°C for 5 min, 35 PCR cycles at 95°C for 1 min, 60°C for 2 min and 72°C for 2 min, were performed, followed by a final extension at 72°C for 7 min. The optimized annealing temperature depended on the primer pair, being 58°C for PSR145. PCR products were electrophoresed on 1.5% agarose gels (Takara, Japan) at 50 V (Mupid-2, CosmoBio, Japan) constant voltage, and visualized with ethidium bromide staining. RFLP probes were kindly supplied by Dr. M.D. Gale (John Innes Centre, UK, PSR clones) and Dr. O. Anderson (USDA, USA, BCD and WG clones).

Cloning and sequencing of PCR products

PCR products amplified with STS primers were sequenced to detect polymorphisms among the three NILs. PCR products were purified with a QIA quick PCR Purification Kit (QIAGEN), and sequenced as described above. Since no polymorphism was detected at three loci, *Xpsr145*, *Xpsr426* and *Xbcd450*, further analysis was carried out only for the locus *Xwg644*.

Chromosomal assignment of PCR products

Five clones were amplified with STS primers (WG644-F1 and WG644-R1) and sequenced for the three NILs. A total of 15 sequences were compared and aligned using CLUSTALW, and were classified into two types differing by the number of (TG) repeats, two or three times as shown in Fig. 1. Chromosomal assignment of these sequences was determined by the analysis of nullitetrasomic lines of "Chinese Spring", by using sequence-specific primers. The $(TG)_2$ type sequence was amplified with primers WG644-F1 and WG644-TG2R (annealing at 54°C, 35 cycles), and the $(TG)_3$-type with WG644-dCAPF and WG644-TG3R (annealing at 60°C, 30 cycles). PCR products were digested with *Hinf*I, by mixing 5 units of enzyme and 1 µl of buffer solution with a 8-µl PCR reaction mixture, and were electrophoresed on a 2.5% Nusieve 3:1 agarose gel (FMC) at 50 V constant voltage.

dCAPS analysis

A dCAPS primer was designed for the analysis of single nucleotide polymorphisms (SNP) in WG644–5B sequences among TD(B) and TD(C), by using dCAPS finder 1.0 (Neff et al. 1998). Nested PCR was performed for dCAPS analysis. The WG644–5B

sequence was firstly amplified by a $(TG)_3$ specific primer, and the PCR reaction mixture was diluted 50 times. A second PCR with dCAPS primers WG644-dCAPF and WG644-dCAPR (annealing at 54°C, 35 cycles) was performed by using 2 µl of the diluted PCR reaction mixture as template DNA. The PCR product was digested with *Nsi*I (3 units) and electrophoresed. Other conditions for PCR, digestion and electrophoresis were the same as described above.

Segregation analysis of *Vrn2* and dCAPS marker WG644–5B

Sixty four BF_2 plants [TD(B)//TD(C)] were cultivated in a glasshouse under 16-h day length conditions with a minimum temperature of 16°C. Segregants which had an unfolded flag leaf within 70 days after sowing were regarded as spring types carrying *Vrn2* in the homozygous or heterozygous condition, and the others as winter types (*vrn2vrn2*). dCAPS analysis was also performed on BF_2 plants, and linkage between *Vrn2* and WG644–5B was investigated.

Results

A single PCR product of the size expected from the RFLP clone sequence (476 bp) was amplified with the STS primer for PSR426. The size of the PCR product of PSR145 and BCD450 was 670 and 990 bp, respectively, being larger than the expected sizes (592 and 896 bp). The existence of introns (78 and 94 bp, respectively) was confirmed by the nucleotide sequence of PCR products. PCR products corresponding to these three RFLP clones were amplified from all of the nulli-tetrasomic lines of the group-5 chromosomes, indicating that these products were not specific to a single chromosome. Furthermore, no sequence polymorphism was detected among a total of 15 clones of PCR products amplified from TD(B), TD(F) and TD(C).

As for WG644, a single PCR product of the expected size (535 bp) was amplified with primers WG644-F1 and WG644-R1. Sequence polymorphism was detected among a total of 15 clones amplified from TD(B), TD(F) and TD(C), and they were classified into three types differing by the number of TG (position 225) and A (position 152) repeats and by a total of 13 base changes (Fig. 1). Among the three types, the $(TG)_2$-type sequence was amplified from all of the nulli-tetrasomic lines for the group-5 chromosomes, by using a $(TG)_2$-specific primer. By contrarst, a PCR product by using a $(TG)_3$-specific primer was absent in nullisomic 5B lines (Fig. 2), indicating that the $(TG)_3$-type sequence originated from the 5B chromosome.

Within the $(TG)_3$-type sequence, a single nucleotide polymorphism specific to TD(B) was detected (C/T, position 158 in Fig. 1). Since this SNP was not involved in a restriction site of any enzyme, dCAPS primer WG644-dCAPR was designed to create a *Nsi*I site (ATGCAT), instead of ATGCTT at position 157–162, in TD(F) and TD(C) (Table 1, Fig. 1). A DNA fragment of 126 bp amplified by nested PCR was digested with *Nsi*I, and a cleaved fragment of 108 bp was detected in TD(F) and TD(C), as expected (Fig. 3). By contrast, a PCR product from TD(B) was not cleaved and a fragment of 126 bp

Fig. 1 Three types of sequence detected among PCR clones amplified by primers WG644-F1 and WG644-R1, and their chromosomal origin. Identities between sequences are indicated by "·" and gaps introduced to optimize alignment by "–". PCR primer sequences are *underlined*

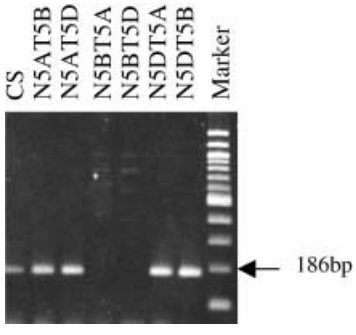

Fig. 2 Amplification of the $(TG)_3$-type sequence of WG644 from nulli-tetrasomic lines of Chinese Spring, by using primer set of WG644-dCAPF and WG644-TG3R. Marker=100 bp DNA ladder

was detected. Similarly, cleaved fragments were detected in other NILs of "Triple Dirk" carrying *vrn2*, TD(D) and TD(E), while uncleaved fragments were detected in "Triple Dirk" carrying *Vrn2* (Fig. 3). Since a non-cleaved fragment was also detected in TD(D), further analysis including the sequencing of the PCR product is required for TD(D). In the BF_2 population [TD(B)//TD(C)] segregation of dCAPS marker WG644–5B was observed (Fig. 4), and the ratio of uncleaved, hetero and cleaved types fitted a 1:2:1 ratio (Table 2, χ^2=1.33).

In the BF_2 population, as shown in Fig. 5, 52 plants unfolded the flag leaf within 70 days after sowing without a vernalization treatment, while 12 plants did not unfold. They were phenotypically judged as spring and winter types, respectively, and the segregation ratio fitted a 3:1 ratio (χ^2=1.84). Spring-type segregants can be also divided into early (49–53 days) and medium (55–

Table 1 Sequences of STS and dCAPS primers used in the present study

Clone name	Primer	Primer sequence (5′→3′)	Annealing (°C)
BCD450	BCD450-F BCD450-R	CATGAATCCGCATTATCATAA AGCTGGGTGTCGTACGCTGG	60
PSR145	PSR145-F PSR145-R2	TCTGAATCTTCATGCAAGATC AGCGACCTGGTCTACGACTGC	58
PSR426	PSR426-F1 PSR426-R1	GGGTTATCTTTCTACTTTCG TTGGACATCACTGATCCTCA	60
WG644	WG644-F1 WG644-R1	TACATCTTCTTTCAGGTCAGC CCCGAATTTACGCACGACAG	60
WG644	WG644-dCAPF WG644-dCAPR WG644-TG2R WG644-TG3R	CTGCTAGCGAGAGAGTTGTGG GAAGACCCGAAAATCGAATGC CTATCTCCTGTGGAGGCACAA ATCTCCTGTGGAGGCACACAA	–[a] –

[a] –; see text

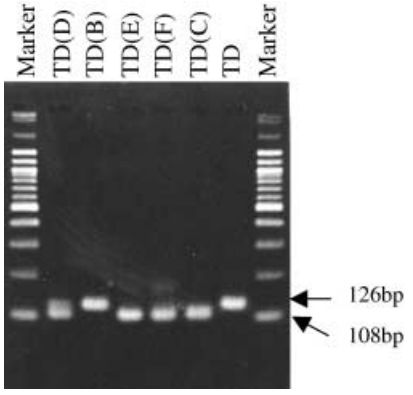

Fig. 3 dCAPS products of WG644-5B from NILs of Triple Dirk. PCR products were amplified by nested PCR and digested with *Nsi*I. Marker=100 bp DNA ladder

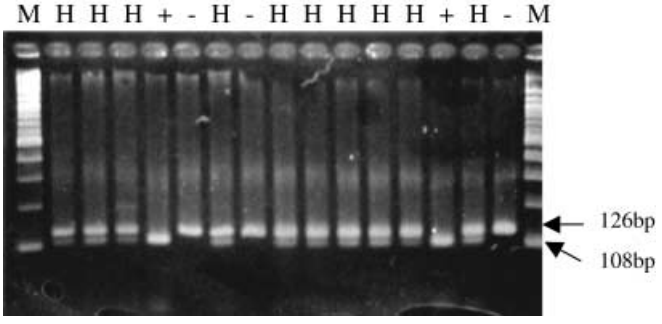

Fig. 4 Segregation of dCAPS marker WG644-5B in a BF_2 population of TD(B)//TD(C). PCR products were amplified by nested PCR and digested with *Nsi*I. +, cleaved type; –, uncleaved type, H Hetero type, M=100 bp DNA ladder

70 days) types based on the days to flag leaf unfolding, whose genotypes can be hypothesized to be *Vrn2Vrn2* and *Vrn2vrn2*, respectively. Segregation of these genotypes was 12:40:12 and fitted a ratio of 1:2:1 (χ^2=4.00). However, the classification of early and medium types was imperfect, and thus the linkage with WG644–5B was analyzed based on their phenotype. As shown in Table 2, χ^2-linkage was statistically significant and the recombination value between *Vrn2* and WG644–5B was 0.017%.

Table 2 Segregation of growth habit and a dCAPS marker in a BF_2 population of TD(B)//TD(C)

Growth habit	WG644-5B[a]			Total	χ^2– linkage
	–	–/+	+		
Spring type	15	37	0	52	
Winter type	0	1	11	12	
Total	15	38	11	64	38.99**

**; Significant at 1%
[a] –, –/+, +; uncleaved hetero, and cleaved type, respectively

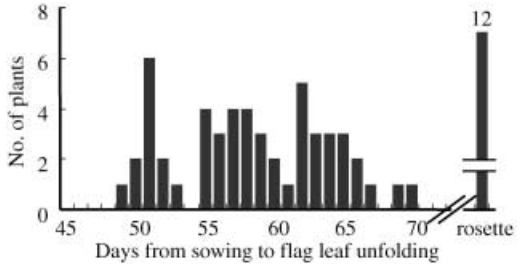

Fig. 5 Frequency distribution of the days from sowing to flag leaf unfolding in a BF_2 population of TD(B)//TD(C)

Discussion

Since the nucleotide sequence at the three orthologous loci of hexaploid wheat is well-conserved, multiple PCR products of the same size are often amplified even by using STS primers (Van Campenhout et al. 1995; Bryan et al. 1999). In such cases, for the development of PCR markers linked to a target gene, a genome-specific sequence must be characterized by sequencing PCR products, and sequence polymorphism must be detected among cultivars. However, the level of sequence polymorphism is as low as 0.001 per nucleotide (Bryan et al. 1999). Also, in the present study, no polymorphism was detected at three loci, *Xpsr145*, *Xpsr426* and *Xbcd450*, all of which corresponded to cDNA clones. By contrast, at *Xwg644* corresponding to a genomic clone, two types of sequence polymorphisms such as SSR polymorphism

and SNP were successfully detected (Fig. 1), and facilitated the development of a specific primer for WG644–5B and a dCAPS primer (Table 1), respectively. A single mismatch was introduced into the dCAPS primer at the third base from the 3′ end (Fig. 1), as indicated by Michaels and Amasino (1998). Nested PCR by using these two primers, followed by a *Nsi*I digest, clearly exhibited polymorphism among the NILs of "Triple Dirk" (Fig. 3).

Based on the orthologous relationship at *Vrn-1*, 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* in the new nomenclature system. To solve such a confusion, in the present study, sequence polymorphism at RFLP loci closely linked to *Vrn-1* was surveyed, resulting in the establishment of a dCAPS marker WG644–5B linked to *Vrn2* by 1.7 cM (Table 2). 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 *Vrn-1* on chromosome 5B, i.e. *Vrn-B1*. Although 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 (Fig. 2). *Xwg644* and *Xpsr426* have also beem mapped on chromosome 5 in other species of *Triticeae*, and *Vrn-A^{m}1* (Dubcovsky et al. 1998), *Vrn-R1* (*Sp1*) (Plaschke et al. 1993) and *Vrn-H1* (*Sh2*) (Laurie et al. 1995) are considered as orthologous genes of *Vrn-1* based on their linkage relationship with these RFLP loci.

The map distance between *Xwg644* and *Vrn-1* has been reported as 0 cM (Galiba et al. 1995; Dubcovsky et al. 1998), 5 cM (Laurie et al. 1995) and 7.5 cM (Korzun et al. 1997). In these studies, segregating populations derived from the cross with a chromosome substitution line of *Triticum spelta* 5A (Galiba et al. 1995) or synthetic wheat (*Aegilops tauschii*×*Triticum durum*, Korzun et al. 1997), between barley cultivars (Laurie et al. 1995), or between *Triticum aegilopoides* accessions (Dubcovsky et al. 1998), were used as mapping populations. NILs of "Triple Dirk", TD(B) and TD(C), have been developed by crossing "Winter Minflor" as a non-recurrent parent to introduce recessive alleles of *Vrn* genes (Pugsley 1971). "Triple Dirk" is known to carry *Vrn-A1* and *Vrn-B1*, and thus the chromosomal segment of TD(C) including *vrn-B1* and WG644–5B should be derived from "Winter Minflor." The recombination value between chromosome segments of "Triple Dirk" and "Winter Minflor" proved to be 1.7%, which is in good accordance with the reported distance.

In addition to *Vrn-1*, other orthologous genes, *Vrn-2* (*Vrn-H2*, *sh*) and *Vrn-3*(*Vrn-H3*, *Sh3*) in barley and *Vrn-4* (*Vrn-B4*, *Vrn5*) in wheat, are known and have been mapped on 4HL, 1H and 7BS chromosomes, respectively (Law 1966; Takahashi and Yasuda 1971; Laurie et al. 1995). In *Triticum monococcum*, the existence of an additional *Vrn* gene was confirmed by fine RFLP mapping, and proved to be an ortholog of *Vrn-2* on chromosome 4 whose segment was translocated to the long arm of 5A^m (Dubcovsky et al. 1998). Although chromosomal location and linkage relationship have not been identified for *Vrn4* carried by TD(F), independence of *Vrn-B1* and *Vrn4* was clearly shown by a test cross between four NILs of "Triple Dirk" (Gotoh 1979; Kato, unpublished), and by the existence of landraces carrying either *Vrn-B1* or *Vrn4* (Iwaki et al. 2001a). Kato et al. (1993) suggested that *Vrn4* is located on chromosome 5D by monosomic analysis. However, *Vrn-D1* has been already mapped on 5DL, and a translocation including chromosome 5D has not been reported. Further analysis is required to clarify the origin and orthologous relationship of *Vrn4*. Furthermore, even in common wheat, local landraces carrying *Vrn* gene(s) other than *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Vrn4*, have been identified in various areas (Iwaki et al. 2000, 2001a). Detailed analysis of these landraces might lead to the identification of orthologous genes, *Vrn-3* and *Vrn-2*, on groups 1 and 4 in wheat.

Vernalization requirement is an important character to avoid frost injury during cold winter, and thus wheat cultivars with a different level of vernalization requirement are cultivated in various areas depending on the growing condition in each area. The analysis of a worldwide collection of wheat landraces clearly showed that spring-type wheats with a partial requirement for vernalization are adapted to areas where the average January temperature ranged from 4°C to 10°C (Iwaki et al. 2000, 2001a). The *Vrn* genotype of spring-type wheats is also different between areas, and carriers of *Vrn-B1* are frequently cultivated in the southern part of Europe. Therefore, the dCAPS marker WG644–5B, developed in the present study, could be applicable to the practical breeding programmes in these areas. From another stand point, for genetical and physiological studies of vernalization requirement, NILs carrying multiple *Vrn* genes with various combinations must be established. However, the pyramiding of *Vrn* genes is practically very difficult, since test crosses must be carried out to identify the *Vrn* genotype of each hybrid progeny. Therefore, PCR-based linkage markers should be developed also for other *Vrn* genes, and then wheat genotypes carrying multiple *Vrn* genes can be easily selected. The analysis of these NILs should make it possible to clarify the epistatic relationships among *Vrn* genes, and give way to the exploration of diversified levels of vernalization requirement in the breeding of spring-type wheats.

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