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ORIGINAL PAPER

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Allelic variation at the linked *AP1* and *PhyC* loci in hexaploid wheat is associated but not perfectly correlated with vernalization response

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Abstract Vernalization requirement is an important trait in temperate crop plants such as wheat and must be considered when selecting varieties for cultivation under different climatic conditions. To determine the growth habit of wheat varieties, plants need to be grown under different vernalization regimes, a lengthy but necessary process for breeders involved in crossing winter with spring germplasm. If haplotypes can be associated with growth habit, then molecular marker assays that are reliable, cheap, and quick can be developed to assist in the selection of plants with the desired phenotype. We have analyzed 81 accessions that have different vernalization requirements and putative different origins of spring habit for sequence variation at the *Apetala1* (*AP1*) locus, which underlies *Vrn-1*, and at the linked *Phytochrome C* (*PhyC*) locus. Good correspondence was found between the *AP1* genotype and the *PhyC* haplotype for 77 of the 81 accessions. Two varieties displayed a recombination event between the *AP1* and *PhyC* loci, and one variety carried a recombinant *PhyC* gene. In addition, one variety carried an apparent *AP1* winter allele, but displayed the *Vrn-A1* spring habit. The *PhyC* haplotype for this variety also indicated the presence of a *Vrn-A1* spring allele. Our data suggest that both the *AP1* promoter region and *PhyC* SNPs can be used as diagnostic markers for vernalization response at the *vrn-A1* locus, but that neither are perfect tags.

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Introduction

Varieties of hexaploid bread wheat, *Triticum aestivum* ($2n = 6x = 42$), can be categorized into winter and spring types based on their vernalization requirement. In winter varieties, flowering is delayed unless plants are exposed to a period of near-freezing temperature. In contrast, spring varieties are largely vernalization insensitive. The *vrn-1* genes are the major genes that determine spring/winter type in hexaploid bread wheat and have been mapped to the long arms of the homoeologous group 5 chromosomes (Pugsley 1971; Snape et al. 1976). Spring growth habit is dominant to winter, so that a spring allele at any of the homoeologous *Vrn-A1*, *B1* or *D1* loci in hexaploid wheat will promote flowering in the absence of vernalization. In European wheat varieties, spring alleles at *Vrn-A1* are predominant in reducing vernalization requirement (Pugsley 1971; Snape et al. 1976) and may have been derived from a single source. An independent source of *Vrn-A1* alleles may be provided by *Triticum spelta*, which exhibits very early flowering in the absence of vernalization (Snape et al. 1976). The *Vrn-A1* *T. spelta* allele is therefore likely to be different from the spring allele prevalent in European spring cultivars.

Phytochromes are photoreceptors that allow plants to monitor light and, in response, to regulate changes in gene expression that underlie responses to the light environment (for a recent review, see Quail 2002). Phytochromes, through their responses to light, have been implicated in the regulation of flowering (Kaczorowski and Quail 2003). In *Arabidopsis*, there are five discrete phytochrome-encoding genes, *PhyA–PhyE*, resulting from four duplication events (Mathews and Sharrock 1996). The first duplication gave rise to the *PhyA*/*C* and *PhyB*/*D*/*E* lineages. The second duplication led to the

formation of *PhyA* and *PhyC*. Monocots have only three phytochrome genes, *PhyA, PhyB*, and *PhyC*. Therefore, the duplications leading to *PhyB*/*D* and *PhyE*, and the split of *PhyB*/*D* most likely occurred in dicots after their divergence from the monocots, although it cannot be excluded that *PhyE* was lost in an early monocot ancestor (Mathews and Sharrock 1996). To evaluate whether the phytochrome genes are underlying any of the major flowering time genes in the Triticeae as had been shown for *PhyB* and the barley BMDR1 and sorghum ma_3^R photoperiod-response mutants (Childs et al. 1997; Hanumappa et al. 1999), the *PhyA, PhyB*, and *PhyC* genes were mapped in wheat, barley, and/or rye (J. Beales, D.A. Laurie, and K.M. Devos, unpublished). None of the *Phy* loci mapped to regions of known photoperiod-response genes, but *PhyC* cosegregated with *vrn-A1* in wheat in a population of 96 doubled haploid lines. To assess whether *PhyC* was a candidate for *Vrn-1*, full-length *PhyC-5A* genes were isolated from the *vrn-A1* (winter allele) varieties 'Chinese Spring' and 'Soleil', and from the *Vrn-A1* (spring) cultivars 'Reward', 'Saitama 27', and *T. spelta* acc. 'Grey' and their sequences compared (K.M. Devos et al., unpublished). We focused on *Vrn-A1* as it is the most widely deployed spring gene in European wheats. The identified *PhyC-5A* polymorphisms were subsequently assessed in a panel of 81 hexaploid winter and spring wheat varieties. Although it is now known that the *Vrn-*A^m *1* gene is a MADS-box transcription factor closely related to the *Arabidopsis APETALA1* (*AP1*) gene, and that *PhyC* is located some 300 kb from *Vrn-*A^m *1* in *Triticum monococcum* (Yan et al. 2003), the *PhyC* haplotype data are presented here as a case study to demonstrate (1) that markers closely linked to a trait can be used effectively as molecular tags and (2) that using associations between polymorphisms and traits to confirm the function of putative candidate genes is not straightforward, at least not in polyploids. In the era of comparative genomics, selection and validation of candidate genes from rice or other grass species for traits in cereal crops of interest represent a shortcut to map-based cloning, and it is important to understand the problems that may be encountered when following the candidate gene route. The *PhyC* haplotype study was complemented with data on the variation present in our sample of hexaploid wheat varieties in the region of the *Vrn-A1* (*AP1-5A*) promoter previously shown to be associated with winter/spring habit in *T. monococcum* (Yan et al. 2003).

Materials and methods

Plant material

Seeds from 81 wheat varieties (Table 1) were obtained from the John Innes Centre (JIC) collection and from the National Small Grains Collection maintained by USDA-ARS, Aberdeen, Idaho. The seeds were germinated on wet filter paper in 9-cm petri dishes at 20°C for 72 h. Five seedlings of each variety were grown for DNA extraction. To test flowering time, six seedlings of each variety were grown in a glasshouse under 16 h daylight/8 h darkness at 20°C. The heading date for each plant was recorded when the first ear was half out of the flag leaf. Average heading dates were derived from the six replicates of each variety. Varieties were classified as spring if they flowered within 2 months of sowing and winter if they had not flowered 107 days after germination. Classification was unambiguous as no plants flowered in the 61–107 days interval after sowing.

SNP analysis

Five forward primers and six reverse primers were designed to flank three single nucleotide polymorphisms (SNPs), a 12-bp deletion, and a miniature inverted-repeat transposable element (MITE) present in the 5′ untranslated region (5′UTR) of *PhyC*, and four SNPs located in exon 1 (Fig. 1). The SNPs, deletion, and MITE had previously been shown to differentiate the *PhyC-5A* alleles of the varieties 'Chinese Spring' (*vrn-A1*), 'Soleil' (*vrn-A1*), 'Reward' (*Vrn-A1*), 'Saitama 27' (*Vrn-A1*), and *T. spelta* acc. 'Grey' (*Vrn-A1*) (K.M. Devos et al., unpublished). Primer sequences are given in Table 2. DNA extractions were carried out as described in Devos et al. (1992). PCR was performed in a 50-µl reaction containing 1× PCR buffer, 0.1 mM dNTPs, 50 ng forward and 50 ng reverse primer, and 1 U *Taq* DNA polymerase (Boehringer Mannheim). PCR amplification was performed with an initial denaturation step of 94°C for 2 min, followed by 30 cycles of 94°C for 50 s, 58°C for 50 s, and 72°C for 90 s.

For SNPs 5 and 6, F2/R2 amplicons from the A, B, and D genomes of wheat were cloned into the pGEM-T Easy Vector (Promega). A-genome clones were selected using the genome-specific primers PCCF36 (5′-TGATATGTGATTGTGCTGCAAG-3′) and PCCR17 (5′-AGGAGCATATCACACAGAAGC-3′). These clones were sequenced from both ends using the Big Dye Terminator, version 3, kit (Perkin Elmer/Applied Biosystems), and fragments were separated on an ABI 3700 (Applied Biosystems). Sequence analysis was carried out using the software packages Staden (Gleeson and Staden 1991) and GCG10 (Wisconsin Package, version 10.1, Genetics Computer Group, Madison, Wis., USA).

To detect the presence of the MITE, F35/R47 products were separated by agarose gel electrophoresis (1.2%) and visualized by staining with ethidium bromide. To analyze SNPs 1, 2, 3, 4, and 7, PCR amplification products were digested with the appropriate restriction enzyme (Table 2) in 10-µl reactions according to the supplier's instructions. Restriction fragments were separated on 5% denaturing polyacrylamide gels and visualized by silver staining. To detect the presence or absence of the 12-bp deletion, the undigested F35/R25

Table 1 Sequence variation within the *PhyC-5A* gene and the *AP1* promoter

Variety	Phenotype	MITE	SNP1	12-bp deletion	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	*PhyC* haplotype	*AP1* allele
'Atou'	Winter	–	C	+	C	G	A	nd[a]	nd	A	I	*AP1-5Aa*
'Beaver'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Brock'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Camp Remy'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'California'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Capelle-Desprez'	Winter	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Cheyenne'	Winter	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Consort'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Dean'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Dwarf A'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Fresco'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Hardi'	Winter	–	C	+	C	G	nd	nd	nd	nd	I	*AP1-5Aa*
'Haven'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Hereward'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Kanred'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Maestro'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Maris Huntsman'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Mercia'	Winter	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Norman'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Orlandi'	Winter	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Orso'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Petit Quinquin'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Produttore'	Winter	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Riband'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Salto'	Winter	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Solaris'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Soleil'	Winter	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'VPM1'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Warden'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Wilhelmina 191269'	Winter	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Chablis'	Spring	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Falcon'	Spring	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Florence'	Spring	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Funo'	Spring	–	C	+	C	G	A	A	A	A	I	*AP1-5Aa*
'Mara'	Spring	–	C	+	C	G	A	nd	nd	A	I	*AP1-5Aa*
'Yeoman'	**Winter**	+	**T**	–	**G**	**C**	**C**	**A**	**A**	**A**	**II**	***AP1-5Aa***
'Axona'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Cadenza'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Cadet'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Conley'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Garnet'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Heines Kolben'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Highbury'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Hope'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Jerico'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Jufy I'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Koga II'	Spring	+	T	–	G	C	C	A	A	A	II	*AP1-5Ab*
'Koto'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Maris Butler'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Maris Dove'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Marquis'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Newthatch'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Opal'	**Spring**	+	**T**	–	**G**	**C**	**C**	**nd**	**nd**	**A**	**II**	***AP1-5Aa***
'Red Fife'	Spring	+	T	–	G	C	C	A	A	A	II	*AP1-5Ab*
'Redman'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Reward'	Spring	+	T	–	G	C	C	A	A	A	II	*AP1-5Ab*
'Ring'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Saitama 27' (USDA)	Spring	+	T	–	G	C	C	A	A	A	II	*AP1-5Ab*
'Saratovskaya 29'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Selkirk'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Sharbati Sonora'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Shiraz'	Spring	nd	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Shortandinka'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Sicco'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Taichung no 32'	Spring	+	T	–	G	C	C	A	A	A	II	*AP1-5Ab*

Table 1 (Contd.)

Variety	Phenotype	MITE	SNP1	12-bp deletion	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	*PhyC* haplotype	*AP1* allele
'Thatcher'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Tonic'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'White Fife'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Wilhelmina 191341'	Spring	+	T	nd	G	C	C	nd	nd	A	II	*AP1-5Ab*
'WW15'	Spring	+	T	–	G	C	C	nd	nd	A	II	*AP1-5Ab*
'Ardito'	Winter	+	C	–	G	G	C	G	C	A	III	*AP1-5Aa*
'Caxton'	Winter	+	C	–	G	G	C	G	C	A	III	*AP1-5Aa*
'Moulin'	Winter	+	C	–	G	G	C	G	C	A	III	*AP1-5Aa*
'Spark'	Winter	+	C	–	G	G	C	G	C	A	III	*AP1-5Aa*
'Haya Komugi'	Spring	+	C	–	G	G	C	G	C	A	III	*AP1-5Aa*
'Saitama 27' (JIC)	**Spring**	+	**C**	–	**G**	**G**	**C**	**G**	**C**	**A**	**III**	***AP1-5Ab***
Triticum spelta acc. 'Grey'	Spring	+	N/A[b]	N/A	N/A	G[c]	C	A	A	G	IV	AP1-5Ac
'Exchange'	Spring	+	T[c]	–	G[c]	G[c]	C	A	A	G	V	AP1-5Ad
'Chinese Spring'	Spring	+	C	–	G	G	C	A	A	A	VI	*AP1-5Aa*
'Taichung no 2'	**Spring**	+	**C**	–	**G**	**G**	**C**	**A**	**A**	**A**	**VI**	***AP1-5Ab***
'Hyper'	Spring	+	T	–	G	G	C	A	A	A	VII	*AP1-5Aa*

Varieties for which *PhyC-5A* and *AP1-5A* genotypes disagree are indicated in *boldface*

[a] *nd* Not determined

[b] *N/A* The 3′ duplication carrying the 12-bp deletion and single nucleotide polymorphisms (SNPs) 1 and 2 is absent in *T. spelta* acc. 'Grey'

[c] The DNA fragment from which the SNP was assayed had a different size

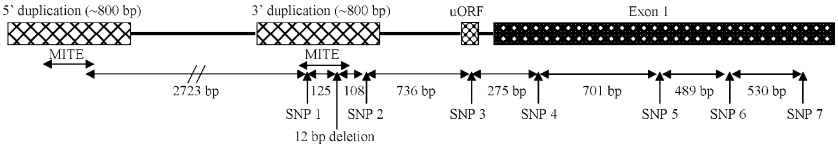

Fig. 1 Relative positions of single nucleotide polymorphisms (*SNP*s), miniature inverted-repeat transposable element (*MITE*) and deletion analyzed in the promoter region and in exon I of *PhyC-5A* in hexaploid bread wheat

products were subjected to 5% denaturing polyacrylamide gel electrophoresis.

Analysis of the *AP1* promoter region

The primers described by Yan et al. (2003) that amplified the region containing a 20-bp deletion in the *AP1* promoter in spring versus winter *T. monococcum* accessions were used to amplify the homoeologous region from the A genome of hexaploid wheat. PCR products were subjected to agarose gel electrophoresis (2%) and visualized by staining with ethidium bromide. The *AP1* fragments amplified from the varieties 'Soleil', 'Reward', 'Exchange', and from *T. spelta* acc. 'Grey' were cloned in the pGEM-T Easy Vector and sequenced.

Results

Haplotypes at the *PhyC-5A* locus

The genotypes at each of the seven SNPs, the presence/absence of a MITE, and of a 12-bp deletion generated seven haplotypes in the set of 81 varieties analyzed (Table 1). Thirty five varieties were classified as haplotype I, 35 as haplotype II, six as haplotype III, one (*T. spelta* acc. 'Grey') as haplotype IV, one ('Exchange') as haplotype V, two as haplotype VI, and one ('Hyper') as haplotype VII. The positions of the SNPs, deletion, and MITE in the *PhyC* gene are shown in Fig. 1. The entire haplotype spans some 5.7 kb.

Thirty of the 35 haplotype I varieties had the winter growth habit, while 34 of the 35 haplotype II varieties had been characterized as spring wheats. The growth habit for the different varieties (Table 1) was either obtained from published pedigree information (http://genbank.vurv.cz/wheat/pedigree/pedigree.asp; http://www.ars-grin.gov/npgs) or based on flowering time in the absence of vernalization. Although we can be sure that varieties that flower within 2 months of sowing in the absence of vernalization carry a spring allele, this method of classification does not allow distinguishing between the presence of dominant spring alleles at the *Vrn-A1, Vrn-B1*, and *Vrn-D1* loci.

Allelic variation at the *AP1-5A* locus in hexaploid wheat

Primers spanning the 20-bp deletion in the *AP1* gene in *T. monococcum*, which was reported to be the cause of the change in phenotype from winter to spring habit (Yan et al. 2003), produced an A-genome-specific fragment in the haplotype I varieties that was slightly larger than that amplified from a *T. monococcum* winter allele.

Table 2 Primer pairs and detection method for the SNPs, deletion, and miniature inverted-repeat transposable element (*MITE*) analyzed in *PhyC-5A*

SNP/insertion	Primers	Primer sequence	Detection method
SNP 1	PCCF35	TCAAAGCCGGGTGATCATC	*Hin*dIII digestion
	PCCR25	CCACTTGATCGGAATAATTGAAG	
SNP 2	PCCF35	TCAAAGCCGGGTGATCATC	*Nla*III digestion
	PCCR25	CCACTTGATCGGAATAATTGAAG	
SNP 3	PCCF13	TCTCCCCCGTCCTTCTCCAG	*Bsl*1 digestion
	PCCR8	AGCGGTTGAGCGCGCTGAC	
SNP 4	PCCF22	TCGGGGGTGGTCGTGGTG	*Bst*F51 digestion
	PCCR5	GTTGTCATCCTGAATGAGCTTC	
SNP 5	PCCF2	TATCTTGGCCTGCACTACCC	Sequencing
	PCCR2	GCATCCATTTCAACATCCTCC	
SNP 6	PCCF2	TATCTTGGCCTGCACTACCC	Sequencing
	PCCR2	GCATCCATTTCAACATCCTCC	
SNP 7	PCCF31	CAACACCCTCTGAAGGAGAG	*Aci*I/*Nsi*I digestion
	PCCR18	ATAGGGGGTATGAGCTCATTG	
MITE	PCCF35	TCAAAGCCGGGTGATCATC	Length polymorphism
	PCCR47	TCGTCCTGGATCGGTTAGGC	
12-bp deletion	PCCF35	TCAAAGCCGGGTGATCATC	Length polymorphism
	PCCR25	CCACTTGATCGGAATAATTGAAG	

Sequence analysis showed that this fragment, referred to as *AP1-5Aa*, differed from the *T. monococcum* winter allele by an 8-bp insertion 35 bp downstream of the putative CArG box (Fig.2). The sequence is identical to that detected in five *vrn-A1* varieties by Yan et al. (2004).

The *AP1-5A* fragments amplified from all but two haplotype II varieties were 231 bp longer than those amplified from haplotype I varieties. The increased size of the *AP1-5Ab* alleles was caused by the insertion of a foldback element, flanked by a TTAAAAACC host duplication, and located 19 bp upstream of the putative CArG box (Fig.2). With the exception of a 1-bp substitution in the foldback element, the *AP1-5Ab* sequence corresponds to the *Vrn-A1a* allele described by Yan et al. (2004). Although the CArG box itself is structurally intact, the hairpin formed by the foldback element may affect the accessibility of the CArG sequence for binding of a repressor molecule, resulting in a spring phenotype. Two haplotype II varieties carried the *AP1-5Aa* allele: 'Yeoman', a winter variety, and 'Opal', a German spring variety classified as having the allele composition *Vrn-A1, Vrn-B1*, and *vrn-D1*.

'Saitama 27' (JIC) was the only haplotype III variety that carried the *AP1-5Ab* allele (Table 1). The other five haplotype III varieties carried *AP1-5Aa*. Of the two haplotype VI varieties one, 'Taichung no 2', carried the *AP1-5Ab* allele while the other, 'Chinese Spring', carried *AP1-5Aa*. *T. spelta* acc. 'Grey' carried the *AP1-5Ac* allele, which differed from the *T. monococcum AP1-5A* winter allele by two single-base pair insertions, one single-base pair deletion, and three base pair substitutions (Fig.2). Several of these are in the proximity of the CArG box and may affect interaction with transcription factors. The variety 'Exchange' carried yet another *AP1-5A* allele, referred to as *AP1-5Ad* (Fig.2). The *AP1-5Ad* allele has a 20-bp deletion 14 bp downstream of the CArG box, two further single-base pair deletions, and one base pair substitution compared to *AP1-5Aa*. The deletions are unique to *AP1-5Ad* and are located in the proximity of the CArG box.

Discussion

In hexaploid wheat, the main locus determining variation in vernalization requirement, *Vrn-1*, is located on the long arms of the group 5 chromosomes tightly linked to the *PhyC* gene (K.M. Devos et al., unpublished). We know from the recent map-based isolation of *AP1*, the gene underlying *Vrn-1*, that the physical distance between *Vrn-1* and *PhyC* is some 300 kb in *T. monococcum* and that, at least in rice, both genes are transcribed in opposite orientation. We have assessed haplotype variation in the *PhyC-5A* and *AP1-5A* genes and correlated the haplotypes with growth habit. Winter habit is prevalent in wild relatives of wheat and spring habit is therefore likely to be the derived growth form.

AP1-5A variation

In no case did we identify a deletion in the *AP1-5A* promoter equivalent to that seen in *T. monococcum* (Yan et al. 2003). This is consistent with an independent origin of spring habit in hexaploid and einkorn wheats. In the present analysis, the insertion/deletion events observed in *AP1-5A* may affect promoter function, but it is unclear whether any are diagnostic of winter or spring habit, because no variant was absolutely correlated with growth habit. This was also the case in the study by Yan et al. (2004), where two landraces were identified out of a sample of 26 varieties with known *Vrn-A1* spring habit that carried the *vrn-A1* sequence over the *AP1* promoter region analyzed. It is conceivable that spring habit has originated more than once, leading to different *AP1* spring alleles in hexaploid wheat. Most of the varieties analyzed in our study came from European breeding programs and were related by pedigree. Nevertheless, our survey also included varieties from the United States, Canada, USSR, Japan, and India, and for some of these varieties, no evidence of intercrossing could be

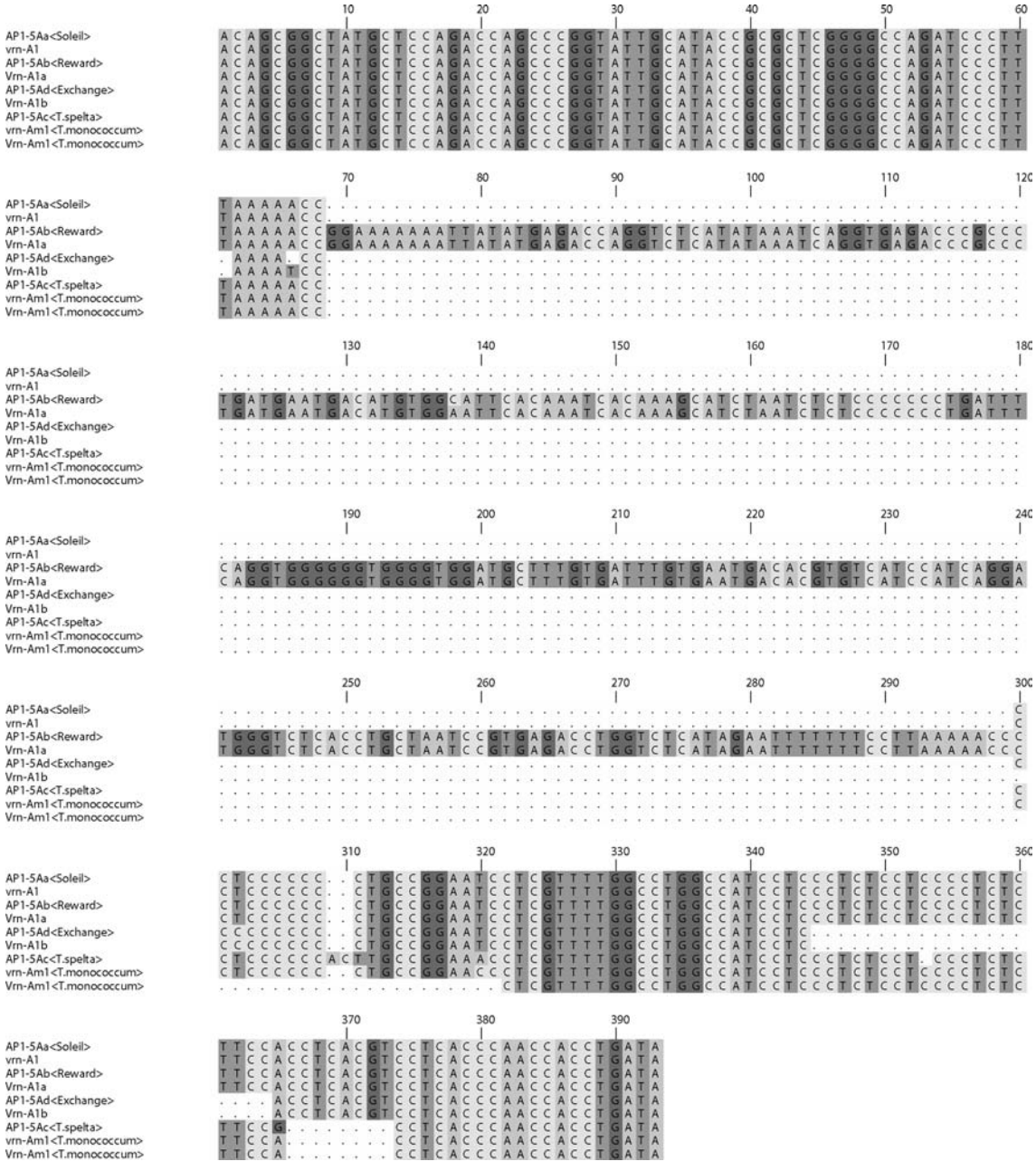

Fig. 2 Sequence alignment of a segment of the *AP1* promoter from the *vrn-A^m 1* and *Vrn-A^m 1 Triticum monococcum* accessions 'G1777' and 'G2528', respectively, from the *Triticum aestivum* varieties 'Soleil' (*vrn-A1*; *AP1-5Aa* allele), 'Reward' (*Vrn-A1*; *AP1-5Ab* allele), and 'Exchange' (*Vrn-A1* phenotype not confirmed; *AP1-5Ad* allele), and from *Triticum spelta* acc. 'Grey' (*Vrn-A1*; *AP1-5Ac* allele). Also included in the alignment are the *vrn-A1*, *Vrn-A1a*, and *Vrn-A1b* alleles identified by Yan et al. (2004) in hexaploid wheat

found based on available pedigree information. The *Vrn-A1* spring varieties 'Reward' and 'Saitama 27', and *T. spelta* acc. 'Grey', and the winter varieties 'Soleil' and 'Chinese Spring', for which the *PhyC-5A* alleles were sequenced, were chosen because of their apparent unrelatedness (K.M. Devos et al., unpublished). The different SNP haplotypes at the *PhyC-5A* locus confirmed that these lines indeed came from independent breeding programs.

Correlation of *PhyC-5A* haplotypes with growth habit

PhyC-5A haplotype I appeared to be characteristic for the presence of a winter allele at the *vrn-A1* locus. Although five haplotype I varieties were classified as spring wheats (Table 1), most likely they carry a winter allele at the *vrn-A1* locus and spring alleles at the *Vrn-B1* and/or *Vrn-D1* loci. The *Vrn-1* allele composition is known only for 'Mara', an Italian wheat, which carries *vrn-A1,*

Vrn-B1 and *vrn-D1* (Xin et al. 1988). 'Falcon' has been reported to carry *Vrn-A1* (Pugsley 1972); however, analysis of the *AP1* promoter region around the CArG box demonstrated that all haplotype I varieties, including 'Falcon', carried the *AP1-5Aa* allele, confirming their winter habit at the *vrn-A1* locus. It is possible that 'Falcon' has a different mutation conferring spring habit in the *AP1-5A* gene, although the SNP haplotype at the *PhyC-5A* locus would argue against the presence of an independently arisen mutation, unless the mutation occurred very recently. Misclassification of 'Falcon' by Pugsley (1972) is also unlikely as the majority of the varieties in the pedigree of Falcon carry *Vrn-A1*. Furthermore, Falcon was shown by Yan et al. (2004) to carry the *AP1-5Ab* allele. The most likely explanation is that the sample we obtained as 'Falcon' was, in fact, a different variety, perhaps 'Falcone', an Italian spring wheat. Another example of potential erroneous annotation is found in 'Chablis', a haplotype I spring wheat that carries the *AP1-5Aa* allele. 'Chablis' is the result of a cross between 'Jerico' and 'Tonic', two haplotype II varieties that carry the spring *AP1-5Ab* allele (Table 1). Therefore, either the pedigree is incorrect, or the sample received as 'Chablis' was mislabeled.

Haplotype II characterized the majority of the spring varieties analyzed (Table 1). One winter variety, 'Yeoman', also carried haplotype II. 'Yeoman', which is the result of a cross between 'Browick', a winter wheat, and 'Red Fife', a spring wheat, carried the winter *AP1-5Aa* allele. This suggests that during the breeding of 'Yeoman', a recombination event took place between the *AP1* and *PhyC* genes. Interestingly, one further haplotype II variety, 'Opal', was shown to carry the winter *AP1-5Aa* allele. Opal has been characterized as a spring wheat with allele composition *Vrn-A1, Vrn-B1*, and *vrn-D1*. This suggests that the spring allele in 'Opal' may have a different origin.

The six haplotype III varieties originate from Japanese germplasm. 'Haya Komugi' and 'Saitama 27', two spring varieties, were bred in Japan, 'Ardito' resulted from a cross with 'Akagomughi', and 'Moulin' has 'Norin-10' in its ancestry. 'Caxton' and 'Spark' originated from crosses with 'Moulin'. 'Ardito', 'Moulin', 'Caxton', and 'Spark' are winter varieties. In Japanese germplasm, the most prevalent spring allele is *Vrn-D1* (Gotoh 1979). Analysis of *AP1-5A* in 'Haya Komugi' indeed showed that this variety carries the winter *AP1-5Aa* allele, indicating winter habit at the *vrn-1* locus and, presumably, spring habit at the *Vrn-D1* locus. 'Saitama 27', however, had been classified by Gotoh (1979) as carrying *Vrn-A1* and the presence of the spring *Vrn-A1* allele was confirmed in our study by *AP1* analysis. Gotoh (1979) stated that the *Vrn-1* spring allele originated from the US variety 'California'. This could not be verified, as the variety 'California' assayed in our study was clearly a winter wheat. However, the assumption is that the *Vrn-1* gene was introduced from a spring variety that carried haplotype II at the *PhyC* locus. Our results therefore suggest that the line leading to 'Saitama 27' has undergone a recombination event between *AP1* and *PhyC*. It should be noted that a second 'Saitama 27' accession, obtained from the USDA small grains collection, carried haplotype II. It is possible that both 'Saitama 27' accessions are selections from the original *Vrn-A1* haplotype II × Japanese *vrn-A1* haplotype III cross.

T. spelta acc. 'Grey' carried a unique *AP1-5Ac* allele and also varied quite considerably from the *T. aestivum* varieties analyzed at the *PhyC* locus. The 3′ duplicated region, which contained SNPs 1 and 2 and the 12-bp deletion, has been deleted (K.M. Devos et al., unpublished). In addition, the fragment carrying SNP 3 was 1 bp shorter in *T. spelta* acc. 'Grey' compared to all but one *T. aestivum* variety analyzed ('Exchange'), and the G at SNP 7 was present only in *T. spelta* and in the *T. aestivum* variety 'Exchange'. The concurrence of the latter two SNPs in 'Exchange' and *T. spelta* acc. 'Grey' would suggest that spring habit in 'Exchange' may have been contributed by a *T. spelta* accession. On the other hand, the duplication that characterizes the upstream region of *PhyC-5A* in the *T. aestivum* varieties analyzed but not in *T. spelta* acc. 'Grey' was present in 'Exchange'. 'Exchange' also carried a unique 35-bp insertion 130 bp downstream of SNP 2.

According to its pedigree, 'Exchange' is the result of a cross between the winter varieties 'Warden' and 'Hybrid-English'. However, 'Exchange' is early flowering in the absence of vernalization and carries a unique *AP1-5Ad* allele. *AP1-5Ad* is more similar in sequence to the winter *AP1-5Aa* than to the *T. spelta AP1-5Ac* allele. In fact, it shares none of the characteristics that make the *T. spelta AP1-5Ac* allele unique. The *AP1-5A* and *PhyC-5A* results suggest that some wheat accession other than or in addition to 'Warden' and 'Hybrid English' was involved in the pedigree of 'Exchange'. This line, possibly a *T. spelta* accession unrelated to 'Grey' contributed spring habit. 'Exchange' therefore represents a third source of spring *Vrn-A1* alleles.

The *AP1-5Ad* allele identified in 'Exchange' is highly similar in sequence to the *Vrn-A1b* sequence identified by Yan et al. (2004) in 'Marquis'. In our analysis, however, 'Marquis' carried the *AP1-5Ab* allele, which is characteristic for *Vrn-A1* spring varieties. 'Reward', the result of a cross between 'Marquis' and 'Prelude', carries the *AP1-5Ab* allele in both our study and the study by Yan et al. (2004). It would be interesting to analyze the haplotype at the *PhyC-5A* locus to investigate the likely origin of the ''Canadian'' 'Marquis' analyzed by Yan et al.

'Taichung no 2', which has 'Saitama 27' as one of its parents, carries the *AP1-5Ab* allele. This suggests that 'Taichung no 2' inherited the *Vrn-A1* locus from 'Saitama 27'. However, the haplotype at the *PhyC* locus is different from that of 'Saitama 27'. No information is available on the second parent, 'Shoawase', so it is unclear whether the haplotype VI was inherited from 'Shoawase'. If this is the case, 'Shoawase' does not have

the haplotype III structure typically seen in Japanese germplasm. Alternatively, 'Taichung no 2' may be the result of a recombination event between the 'Saitama 27' and 'Shoawase' *PhyC-5A* alleles. The latter is the most likely explanation, in particular if the 'Saitama 27' parent had the haplotype II genotype. Assuming that the transcription orientation of the *AP1* and *PhyC* genes is the same in wheat as in rice, *AP1* is located 3′ to *PhyC*. A recombination event in the region between SNPs 3 and 5 in the *PhyC-5A* genes of 'Saitama 27' (*AP1-5Ab*, haplotype II) and 'Shoawase' (*AP1-5Aa*, haplotype III) would have resulted in the presence of the *AP1-5Ab* allele and haplotype VI. The same *PhyC-5A* haplotype VI was found in 'Chinese Spring', but in combination with an *AP1-5Aa* allele. Chinese Spring is known to carry the *Vrn-D1* allele (Pugsley 1972). This genotype may again be the result of recombination, this time between *AP1-5Aa*–haplotype III and *AP1-5Aa*–haplotype I varieties (Table 1). Haplotype VII, identified in the US spring variety 'Hyper', differs from haplotype II, the standard haplotype for European spring wheats, at SNP 3 and from haplotype III, the haplotype that characterizes Japanese germplasm, at SNP 1. 'Prelude', one of the parents of 'Hyper', is known to carry the *Vrn-A1* allele. The other parent, 'Pacific Bluestem', is an Australian spring variety with unknown allele composition at the *Vrn-1* locus. The presence of an *AP1-5Aa* allele in 'Hyper' would suggest that it carries the winter *vrn-A1* allele. If the 'Hyper' haplotype arose through recombination, we would have to assume that 'Pacific Bluestem' carried haplotype III and had Japanese germplasm in its ancestry.

Conclusions

Allelic variation in the promoter region of *AP1-5A*, the gene underlying *Vrn-A1*, and across *PhyC-5A*, which is located some 300 kb (in *T. monococcum*) from *AP1-5A*, was analyzed in a sample of 81 hexaploid wheat varieties and correlated with winter/spring growth habit. The *AP1-5Aa* and *PhyC-5A* haplotypes I and III are largely associated with *vrn-A1* winter wheats, with *PhyC-5A* haplotype I being characteristic for European *vrn-A1* wheats and haplotype III for *vrn-A1* wheats bred in Japan. *AP1-5Ab* and *PhyC-5A* haplotype II are largely associated with *Vrn-A1* spring wheats. However, neither the *AP1-5A* nor the *PhyC-5A* markers are perfect tags for growth habit at the *Vrn-A1* locus. Three *Vrn-A1* spring varieties, one in our study and two in that of Yan et al. (2004), were identified that contained an *AP1-5A* allele that could not be distinguished from the winter *AP1-5Aa* allele by the analyses methods used. This suggests that mutations in *AP1-5A* promoter (or genic) regions other than that investigated may be responsible for conferring spring habit. These varieties may therefore be good candidates for sequencing the entire *AP1* gene. Despite the physical proximity of the *AP1* and *PhyC* genes, three likely recombination events were identified in the *AP1-PhyC* region. 'Yeoman' and 'Saitama 27' appeared to have undergone a recombination event between *PhyC* and *AP1*, and 'Taichung no 2' contained a recombinant *PhyC* gene.

Overall, the *AP1-5A* promoter and *PhyC-5A* assays used in our study misclassified one and three varieties, respectively, for growth habit at the *Vrn-A1* locus. Therefore, although not perfect, the *AP1-5A* promoter assay provides the best diagnostic tool available to date for identifying the presence of winter or spring alleles at the *Vrn-A1* locus. Our data does not allow us to estimate the extent of linkage disequilibrium (LD) in the *AP1–PhyC* region because of the relatedness of many of the varieties tested. However, considering the good correspondence between the *PhyC-5A* haplotypes and the allele composition at the *Vrn-A1* locus, it would appear that LD extends over a few hundred kilobases, at least in that particular region of chromosome arm 5AL. Considering that wheat is an inbreeding species that has gone through a bottleneck during the polyploidization process, a relatively high level of LD can be expected. Haplotype analysis of SNPs present in genes located within a physical distance of a few hundred kilobases from the target trait therefore provides an alternative to use of the gene itself in marker-assisted selection, in particular when the gene underlying the trait has not yet been isolated or has been patented.

Our study also reveals a number of problems that can be encountered in association analyses. Establishing the allelic composition underlying a phenotype is not straightforward in a recent polyploid such as wheat and will be a limiting factor in large-scale studies. It may also be difficult to get conclusive evidence from association studies that a candidate gene is underlying a trait, unless haplotypes at all three homoeologous locations can be assessed. Inaccuracies in pedigree information and in varietal labeling also pose a problem, in particular when relying on published trait data in the association studies. Haplotype analysis at multiple linked SNPs can, however, reveal (new) information on the origin of germplasm. Despite the drawbacks, association analysis remains a very useful way to identify diagnostic markers for traits.

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References

Childs KL, Miller FR, Cordonnier-Pratt MM, Pratt LH, Morgan PW, Mullet JE (1997) The sorghum photoperiod sensitivity gene, Ma_3, encodes a phytochrome B. Plant Physiol 113:611–619

Devos KM, Atkinson MD, Chinoy CN, Liu C, Gale MD (1992) RFLP based genetic map of the homoeologous group 3 chromosomes of wheat and rye. Theor Appl Genet 83:931–939

Gleeson TJ, Staden R (1991) An X-Windows and UNIX implementation of our sequence-analysis package. Comput Appl Biosci 7:398–398

Gotoh T (1979) Genetic studies on growth habit of some important spring wheat cultivars in Japan, with special reference to the identification of the spring genes involves. Jpn J Breed 29:133–145

Hanumappa M, Pratt LH, Cordonnier-Pratt MM, Deitzer GF (1999) A photoperiod-insensitive barley line contains a light-labile phytochrome B. Plant Physiol 119:1033–1039

Kaczorowski KA, Quail PH (2003) Arabidopsis *PSEUDO-RESPONSE REGULATOR7* is a signaling intermediate in phytochrome-regulated seedling deetiolation and phasing of the circadian clock. Plant Cell 15:2654–2665

Mathews S, Sharrock RA (1996) The phytochrome gene family in grasses (*Poaceae*): a phylogeny and evidence that grasses have a subset of the loci found in dicot angiosperms. Mol Biol Evol 13:1141–1150

Pugsley AT (1971) A genetic analysis of the spring-winter habit of growth in wheat. Aust J Agric Res 22:21–31

Pugsley AT (1972) Additional genes inhibiting winter habit in wheat. Euphytica 21:547–552

Quail PH (2002) Photosensory perception and signalling in plant cells: new paradigms? Curr Opin Cell Biol 14:180–188

Snape JW, Law CN, Worland AJ (1976) Chromosome variation for loci controlling ear emergence time on chromosome 5A of wheat. Heredity 37:335–340

Xin Z-Y, Law CN, Worland AJ (1988) Studies of the effects of the vernalization responsive genes on the chromosomes of homoeologous group 5 of wheat. In: Miller TE, Koebner RMD (eds) Proceedings of the 7th international wheat genetics symposium. IPSR, Cambridge, pp 675–680

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene *VRN1*. Proc Nat Acad Sci 100:6263–6268

Yan L, Helguera M, Kato K, Fukuyama S, Sherman J, Dubcovsky J (2004) Allelic variation at the *VRN-1* promoter region in polyploid wheat. Theor Appl Genet 109:1667–1686