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A PCR Marker for Growth Habit in Common Wheat Based on Allelic Variation at the *VRN-A1* Gene

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ABSTRACT

Growth habit varies among accessions of hexaploid wheat (*Triticum aestivum* L.). The winter habit genotype (homozygous recessive for *vrn-A1*, *vrn-B1*, *vrn-D1*) requires a cold treatment to induce flowering and is generally planted in the fall. Spring habit genotypes flower in the absence of a vernalization treatment. Spring habit is conferred by a dominant allele at any of the three *VRN-1* loci, but varieties carrying the *VRN-A1* locus are the most frequent and usually flower earlier. To facilitate selection in populations segregating at the *VRN-A1* locus and to assist in genotyping wheat accessions of unknown *VRN-1* composition, a PCR-based marker for growth habit would be useful. The *VRN-A1* gene was recently cloned allowing for the development of markers for growth habit. In this report, we designed primer sets specific for the *VRN-A1* locus, and used them to amplify an 810-bp segment of the *VRN-A1* gene between intron 4 and exon 8 from 40 spring wheat lines and 37 winter wheat lines. Three diagnostic nucleotide changes were identified that differentiated the dominant *Vrn-A1* and recessive *vrn-A1* alleles in 75 out of the 77 genotypes analyzed in this study. One of these differences resulted in the presence of an additional *Aci*I restriction site in the allele for spring growth habit. This difference was exploited to develop a cleavage amplification polymorphic sequence (CAPS) that can be used to determine the allelic state at *VRN-A1* in germplasm collections, and as a marker in backcross breeding projects. An additional pair of dominant markers was developed from a different polymorphism.

Wheat primarily exists as either winter or spring growth habit. Winter wheat requires a vernalization period (4–6 wk at temperatures below 10°C) to induce flowering, while spring wheat flowers without a vernalization treatment. Although there are several genes influencing vernalization treatment (McIntosh et al., 2003), the most common difference between spring and winter wheat genotypes in North America is at the *VRN-A1* locus. In common wheat, spring habit is inherited as a dominant trait and winter habit is recessive (Flood and Halloran, 1986).

Spring and winter wheat are grown interchangeably in many regions of the Great Plains, and as a result, breeding criteria for new varieties are similar. However, the two habits have formed isolated gene pools since new spring and winter varieties tend to be developed from varieties of similar habit. In this regard, Barrett and Kidwell (1998) assessed genetic diversity using amplified fragment length polymorphisms (AFLP) for several classes of wheat grown in the Pacific Northwest. Their results showed a clear separation of spring and winter wheat, which was supported by pedigree information (Barrett et al., 1998). Since genetic variance for agronomic traits among progeny lines was correlated with AFLP diversity of parents (Burkhamer et al., 1998), more progeny diversity would be expected in winter by spring crosses.

Several breeding programs have been involved in producing winter × spring crosses. These programs have been interested in increasing genetic diversity and improving certain traits such as yield, winter hardiness, earliness, and drought resistance (Gill et al., 1977; McCuistion, 1978; Kant et al., 2001). Motives for crossing between spring and winter wheat germplasm pools not only include improving diversity for quantitative traits but also include backcrossing for specific genes. In both cases, the inability to determine progeny genotypes easily and reliably at *VRN-A1* is a limitation. This is especially true in backcrossing programs, where distinguishing homozygous and heterozygous spring wheat progeny is not possible. A molecular marker for vernalization habit would be valuable in crossing programs involving both growth habits.

A *VRN-A1* marker also would be useful in genotyping spring germplasm. It is often the case that *VRN-A1* genotype is not known in untested germplasm materials, as spring habit may be due to dominate alleles at other *VRN* loci (McIntosh et al., 2003). Dominant spring alleles at these loci result in genotypes that do not require cold treatment to flower but have differences in flowering time. The effect of the different *VRN-1* genes on flowering is difficult to distinguish from other genes that also influence flowering time in wheat (e.g., response to photoperiod). A simple distinguishing test to determine allelic state at *VRN-A1* would be useful in applied wheat breeding programs.

The *VRN-A1* vernalization locus has been mapped in *Triticum monococcum* L. and associated with RFLP markers (Dubcovsky et al., 1998). *VRN-A1* was also mapped in the deletion-bin maps of *T. aestivum* (Sarma et al., 1998; Sutka et al., 1999). These mapping projects identified RFLP markers that were tightly linked to the *VRN-A1* locus. Unfortunately, RFLPs often show low levels of polymorphism between improved cultivars (Bryan et al., 1999). Iwaki et al. (2002) converted an RFLP marker linked to the orthologous *VRN-B1* locus on chromosome 5B into a dCAPS marker. RFLP markers linked to vernalization also have been used to identify BAC clones in barley, rice and *T. monococcum* and to clone the *VRN-A1* using a map-based cloning approach (Dubcovsky et al., 2001; Yan et al., 2003).

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Abbreviations: CAPS, cleavage amplification polymorphic sequence; CS, Chinese Spring; indel, insertion–deletion; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

The positional cloning showed that wheat *APETALA1* (*AP1*) homolog was the *VRN-A1* gene in diploid wheat, and this was confirmed by expression studies during vernalization (Yan et al., 2003). Trevaskis et al. (2003) and Danyluk et al. (2003) confirmed a similar expression profile of *AP1* in common wheat. The *VRN-A1* sequence information obtained from the previous studies provides the opportunity to develop a PCR marker within the gene for the spring versus winter allele. That opportunity is the focus of this paper.

MATERIALS AND METHODS

Plant Materials

Triple Dirk isogenic line carrying the *VRN-A1* allele for spring growth habit and recessive *vrn-B1* and *vrn-D1* alleles for winter growth habit at the other loci was provided by R.E. Allan at Washington State University. Triple Dirk C is an isogenic winter line with recessive *vrn-1* alleles at all three loci. (Pugsley, 1971; Stelmakh, 1987; Storlie et al., 1998). All other accessions were obtained from the USDA National Small Grains Collection (Aberdeen, ID) or from Montana breeding lines (Table 1). All plant materials were maintained in a greenhouse where temperature ranged from 18 to 25°C with a daylength of 16 h. Spring wheat accessions of unknown genotype were crossed with the Triple Dirk line with the *VRN-A1* allele. Resulting F_2s were tested for growth habit by growing 96 individuals from each cross under greenhouse conditions without vernalization.

Molecular Protocols

Genomic DNA was isolated from 40 spring wheat lines and 37 winter wheat lines as described by Riede and Anderson (1996). Each individual was maintained in the greenhouse to confirm growth habit.

Sequence of *VRN-A1* was available from *T. monococcum* BAC 231A16 (GenBank AY188331, Yan et al., 2003). A probe for the *VRN-A1* gene excluding the conserved MADS-box domain was amplified from BAC 231A16 by means of primers TmExon4-F and Tm3′UTR-R (Table 2). This probe was used to screen the BAC library of the tetraploid variety Langdon (Cenci et al., 2003) and a partial *Eco*RI BAC library of the D genome from *Aegilops tauschii* subsp. *strangulata* (Eig.) Tzvelev AL8/78-2-2 from Armenia (J. Dvorak, M.-C. Luo, and H.B. Zhang, unpublished). The PCR products amplified from the A, B, and D-genome BACs were sequenced, and the intergenome polymorphisms were used to design A-genome specific primers.

The A-genome specific primers VA1-F and VA1-R (Table 2) were used to amplify the distal part of the *VRN-A1* gene from spring and winter wheat lines (Table 1). PCR conditions were as described by Talbert et al. (1994) with annealing temperature of 55°C. The genome-specific PCR products from 40 spring lines and 37 winter lines were cleaned by Wizard PCR preps (Promega, Madison, WI) and cloned into pGEM-T vector (Promega) by means of TOP10F′ (Invitrogen, Carlsbad CA) competent cells. Plasmids with the correct inserts were isolated with Wizard SV Plus Minipreps (Promega) and sequenced in both directions using the T7 and SP6 primers. Sequences were aligned using ClustalW (http://workbench.sdsc.edu; verified 27 April 2004).

Allele-specific primers for the spring and winter alleles of *VRN-A1* (361S-F and 361W-W, Table 2) were labeled with IRDye800 (LI-COR, Lincoln, NE) for use with IR2 DNA Analyzer (LI-COR).

RESULTS

Development of A Genome Specific Primers

Twelve positive BAC clones were identified in the screening of the tetraploid BAC library of Langdon (Cenci et al., 2003) by hybridization with the *VRN-1* specific probe. These twelve BACs were assembled into two groups by *Hin*dIII fingerprinting, and then confirmed by Southern blot hybridization with the *VRN-1* probe. Langdon BACs 1256C17 and 1225D16 were selected to represent each of the two different groups from Langdon, and BAC clone 22J2 was selected from the partial D genome BAC library. Genomic sequences between exon 4 and the end of the genes were obtained by means of primers TmExon4-F and Tm3′UTR-R (Table 2) from BACs 1256C17, 1225D16, and BAC 22J2 (GenBank AY466448, AY466447, AY466446). BAC 1256C17 sequence was the more similar to the A genome of *T. monococcum* (AY188331) indicating that BAC 1256C17 corresponded to the A genome and BAC 1225D16 to the B genome. This genome assignment was confirmed by amplification of nulli-tetrasomic lines from homeologous group five using primers specific for the BAC 1256C17 sequence (Fig. 1, see below).

Comparisons among these sequences revealed numerous differences among the three genomes, which were used to design genome specific primers. The *VRN-A1*-specific forward primer VA1-F was located in intron 4. The sequence of this primer differed from the B and D genome sequences at positions 20 and 25 (Table 2, underlined). The *VRN-A1*-specific reverse primer VA1-R was located in the 3′UTR. The sequence of this primer differed from the B genome sequence at positions 10 and 20 (Table 2, underlined), but only at position 20 from the D genome.

The *VRN-A1*-specific primers amplified an 810-bp region in Chinese Spring and nullisomic-tetrasomic lines lacking chromosomes 5B and 5D, but not in the line lacking chromosome 5A, confirming the A-genome specificity of these primers (Fig. 1).

Polymorphisms Differentiating Alleles for Spring and Winter Growth Habit

VRN-A1-specific primersVA1-F and VA1-R were used to amplify, clone, and sequence the 0.8-kb fragment from the *VRN-A1* gene from different spring and winter accessions (Table 1). To confirm that the cloned products were from the A-genome copy before sequencing, the PCR products from the clones were digested with *Hinf*I. This restriction enzyme cuts only once within the *VRN-A1* gene but twice within the *VRN-B1* and *VRN-D1* genes.

Comparison of the *VRN-A1* sequences from 40 accessions with spring growth habit and 37 accessions with winter growth habit showed two main haplotypes that differed in two 1-bp indels and one single nucleotide polymorphism (SNP, Fig. 2). Other sequence differences were observed in some of the lines, but were not consistent among growth habit groups. Since only one clone from each accession was analyzed, these differ-

Table 1. List of spring and winter accessions comparing reported habit with *VRN-A1* intron sequence.

Accession	Plant name	Location	Habit	Intron†	*VRN-A1* allele‡
CItr6001	BARLETA	Argentina, La Pampa	S	s	D
PI113489	GULAR	Australia, New South Wales	S	w	R
PI348918	RED FIFE	Australia, Victoria	S	s	D
PI113939	WARDEN	Australia, Victoria	S	s	D
CItr1517	Ghirka	Belarus	S	s	D
CItr12019	FRONTEIRA	Brazil, Rio Grande do Sul	S	s	D
PI90834	MARQUIS	Canada, Ontario	S	s	D
CItr15090	HARD RED CALCUTTA	Canada, Saskatchewan	S	s	D
CItr12157	S615	Canada, Saskatchewan	S	s	D
CItr14210	IZOBAMBA 4777	Colombia	S	s	D
CItr12100	EGYPT NA101	Egypt	S	w	R
PI191377	CRETICUM	Ethiopia	S	s	D
CItr5878	KOTA	Former Soviet Union	S	s	D
PI286002	SAUMUR DE MARS	France	S	s	D
PI572916	T. timopheevii	Georgia	S	s	D
CItr14319	INDIA HYBRID 65	India	S	s	D
PI182565	HAYA KOMUGI	Japan, Fuluoka	S	s	D
PI326303	SHORTANDINKA	Kazakhstan, Aqmola	S	s	D
PI182077	S-1	Pakistan, Sind	S	w	R
PI40941	Erinaceum	Pakistan,Punjab	S	s	D
PI192180	MOURISCO	Portugal	S	s	D
PI412959	CERES	South Africa, Cape Province	S	s	D
CItr4735	STEINWEDEL	South Australia	S	s	D
PI348488	T.a. spelta.664	Spain	S	s	D
PI348487	T.a. spelta.663	Spain	S	s	D
PI348488	T.a. spelta.673	Spain	S	s	D
PI348654	T.a. spelta.837	Spain	S	s	D
PI351493	EXTRA KOLBEN I	Sweden, Malmohus	S	s	D
PI90832	KANRED	USA, KS	S	s	D
PI184584	MARQUILLO	USA, MN	S	s	D
PI168659	THATCHER	USA, MN	S	s	D
	Hank	USA, MT	S	s	D
PI549275	Hi-Line	USA, MT	S	s	D
PI574642	McNeal	USA, MT	S	s	D
PI632252	Outlook	USA, MT	S	s	D
PI633974	Chotaeu	USA, MT	S	s	D
CItr17430	Newana	USA, MT	S	s	D
PI607557	Scholar	USA, MT	S	s	D
PI592761	Ernest	USA, ND	S	s	D
	Chinese Spring		S	s	R
	Triple Dirk C		W	s	R
PI366834	XWH1013	Afghanistan	W	w	R
PI410873	Val	Belgium	W	w	R
PI436203		Chile	W	w	R
PI519282		Chile	W	w	R
PI447042	Armada	England	W	w	R
PI452120	Camp Remy	France	W	w	R
PI519205		France	W	w	R
PI573733	Festival	France	W	w	R
PI573739	Tenor	France	W	w	R
PI573742	Chopin	France	W	w	R
PI573746	Forby	France	W	w	R
PI573750	Milpain	France	W	w	R
PI572665		Georgia	W	w	R
PI518790		USA, CO	W	w	R
PI518797		USA, CO	W	w	R
PI518799		USA, CO	W	w	R
	GA8608-D15-7	USA, GA	W	w	R
PI61966	BigSky	USA, MT	W	w	R
PI586806	Nuwest	USA, MT	W	w	R
	Nuwest/MT88001//Mt7865	USA, MT	W	w	R
CItr17844	Redwin	USA, MT	W	w	R
	Rego/CNN39-4-6	USA, MT	W	w	R
PI517194	Tiber	USA, MT	W	w	R
	237	USA, MT	W	w	R
	NuSky	USA, MT	W	w	R
PI564790	95E594	USA, OR	W	w	R
	OR920071	USA, OR	W	w	R
	OR870045	USA, OR	W	w	R
	OR900440	USA, OR	W	w	R
PI1564816		USA, OR	W	w	R
PI519291		USA, OR	W	w	R
	SEG9510280	USA, OR	W	w	R
	SEG9510484	USA, OR	W	w	R
	WA7697	USA, WA	W	w	R
CItr11673	COMANCHE	USA, KS	W	w	R
PI572651	Galliaaralskaja	Uzbekistan	W	w	R

† s = "s" allele, w = "w" allele, see Fig 2.
‡ D = *VrnA1VrnA1*, *R* = *vrnA1vrnA1*.

Table 2. *VRN–1* primer sequences. Polymorphic bases are underlined.

Primer name	Specificity	Sequence
TmExon4-F	None	5′TCT CAT GGG AGA GGA TCT TGA 3′
Tm3′UTR-R	None	5′CAA GGG GTC AGG CGT GCT AG 3′
VA1-F	A genome	5′TCA GAT TCT AGA CTG AGA TGT TCA A 3′
VA1-R	A genome	5′GAT GTG GCT CAC CAT CCA CG 3′
361S-F†	Spring (*Vrn-A1*)	5′AAG CCC GTT ATA TCA CCT TA 3′
361W-F†	Winter (*vrn-A1*)	5′AAG CCC GTT ATA TCA CCT TT 3′

† Allele specific primers 361SF and 361WF are combined with A-genome specific primer VA1-R.

ences could be sequencing artifacts. Conversely, the differences composing the two haplotypes were seen in all accessions. All the accessions with a winter growth habit, except Triple Dirk C, had the same haplotype (Fig. 2, top), which will be referred to hereafter as the "w" haplotype. Thirty-seven out of the 40 accessions with a spring growth habit have the alternative haplotype (Fig. 2, bottom), which will be referred to hereafter as the "s" haplotype. Lines Egypt NA 101, Gular, and S-1 were confirmed to have a spring growth habit, but have the w haplotype (Table 1). Spring growth habit may be due to dominant alleles at other vernalization loci (Flood and Halloran, 1986). To determine the *VRN-A1* genotype for these three accessions, they were each crossed with the tester line Triple Dirk homozygous for the dominant *VRN-A1* allele. If the lines had a spring allele at a *VRN* locus other than *VRN-A1* then a portion of the F_2 progeny would have a winter growth habit, whereas if these three lines had the dominant *VRN-A1* allele, then all the progeny would have a spring growth habit. In each of the three cases tested, a portion of the F_2 progeny were winter. Therefore, Egypt NA 101, Gular, and S-1 are homozygous recessive for *vrn-A1* and their spring growth habit was determined by a dominant allele at one of the other vernalization genes. Chinese Spring was an exception to the association between the dominant *VRN-A1* allele and the s haplotype among the accessions with a spring growth habit (Table 1). Chinese Spring had the s haplotype but is known to carry a recessive *vrn-A1* allele. Its spring growth habit is determined by a dominant *VRN-D1* allele.

Codominant Cleavage Amplification Polymorphic Sequence (CAPS) Marker

The lines with the w haplotype have a T at position 457, whereas the lines with the s haplotype have a C at this position generating an additional recognition site for restriction enzyme *Aci*I (Fig. 2). The s haplotype has five recognition sites for *Aci*I and the w haplotype four (Fig. 2). Digestion of the PCR product from *VRN-A1*-specific primers VA1-F and VA1-R with *Aci*I resulted in a 532-bp fragment in the accessions with the 'w' haplotype and in a 456-bp fragment in the accessions with the s haplotype (Fig. 3).

Allele-Specific Dominant Markers

The two-step procedure required for the CAPS marker and the additional cost of the restriction enzyme could be a limitation in high throughput screenings in breeding programs. For these cases, allele specific primers were designed based on the 1-bp indel at position 361 (Fig. 2). Primer 361S-F had an A at the 3′ end of the primer that matched the 's' haplotype, whereas primer 361W-F had a T at the 3′ end, characteristic of the w haplotype (Table 2). Each of these two primers in combination with the A specific primer VA1-R produced a dominant marker for each of the two different haplotypes.

Since the allele-specific primers differ only in 1 bp they might require optimization of the PCR conditions. During the optimization process, the PCR products can be digested with *Hin*fI to confirm the A-genome origin of the amplification product (no *Hin*fI site within this A genome segment) and with *Aci*I to confirm the amplified haplotype (s haplotype: 116 + 264 bp, w haplotype: 360 bp). Figure 4 shows the PCR amplifications of DNAs from 20 spring (1–20) and 10 winter (21–30) F_2 plants from the cross Newana × Redwin. The PCR products were amplified with the spring haplotype-specific primer 361S-F/VA1-R and then digested with restriction enzyme *Aci*I. The presence of the 116-bp band confirmed that the PCR product was from the s haplotype. No PCR products were detected in the winter accessions.

DISCUSSION

Perfect markers, which show no recombination with the phenotype, have been designed for the dwarfing, hardness, and waxy loci in wheat (Ellis et al., 2002; Gioux and Morris, 1997; Tranquilli et al., 1999; McLauchlan et al., 2001). In these cases, the perfect marker is for the sequence difference that causes the different phenotypes. The advantage of perfect markers is that they are completely linked to the phenotype.

Two out of the 77 accessions analyzed here did not

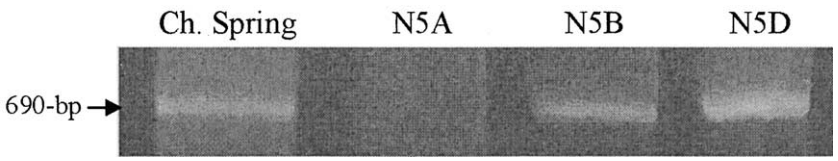

Fig. 1. PCR amplification of primers VA1-F and VA1-R. DNAs were digested with restriction enzyme *Hin*fI. Normal Chinese Spring in the first lane is followed by nulli-tetrasomic lines missing chromosomes 5A, 5B, and 5D, respectively. Note that there is no amplification product in the nulli-5A line, confirming that the primers are A-genome specific. The band size after restriction with *Hin*fI was 690 bp as predicted by the A genome sequence.

```
'w'  TCAGATTCTAGACTGAGATGTTCAAAATATGTATATGCATTTTAGTCATATGCTCTTCA  59
's'  ..........................................................
'w'  TAGTTAAAAAAAAATGACTAATTTTTTTCATTTTTTGTACTTGCAGAACCAACTTATGCA  118
's'  ...........-..........................................................
'w'  CGAATCCATTTCTGAGCTTCAGAAGAAGGTAAGCTGTCAACCTTGCATACCTTATTCGG  177
's'  ..........................................................
'w'  TATTCGAACTGGTCAACTTGTCATGAAGCCTTAGCTTGTTTCAAGATTTGTGACATTAT  236
's'  ..........................................................
'w'  AACATGTATGCAAGTAACTGGTCTACATGCACGTAACCTCATTACATCGTTCTTGCTGC  295
's'  ..........................................................
'w'  AGGAGAGGTCACTGCAGGAGGAGAATAAAGTTCTCCAGAAGGAAGTAAGCCCGTTATAT  354
's'  ..........................................................
'w'  CACCTT-TGGTCCAACCGGTCTAAATTGTTCCGTATAGCAAATTTTATTGACAGAGGTC  413
's'  ......A...................................................
'w'  CGTGTCCCTTCCCCACAGCTCGTGGAGAAGCAGAAGGCCCATGTGGCGCAGCAAGATCA  472
's'  ..........................................C..............
'w'  AACTCAGCCTCAAACCAGCTCTTCATCTTCTTCCTTCATGCTGAGGGATGCTCCCCCTG  531
's'  ..........................................................
'w'  CCGCAAATACCAGGTGATGATGTACATCACAAGTCTAATCTTATTCAGAGTTCAAGTAA  590
's'  ..........................................................
'w'  CCATCTTTTGAATTGGTCGGGTTGTTCCTTGCAGCCCACTTTTGGTCTCTATGCAGTTC  649
's'  ..........................................................
'w'  TGTCGGGCCACATTTAAGTAACATAATACTAATATGCTTGGGTTCGCTTTGGGTTGTGC  708
's'  ..........................................................
'w'  AGCATTCATCCAGCGGCAACAGGCGAGAGGGCAGAGGATGCGGCAGTGCAGCCGCAGGC  767
's'  ............____.....................____.........____....
'w'  CCCACCCCGGACGGGGCTTCCACCGTGGATGGTGAGCCACATC
's'  ..........................................  810
```

Fig. 2. Consensus sequences for winter and spring genotypes of the region amplified by *VRN-A1*-specific primers (indicated in bold-italic letters at the beginning and end of the sequence). Dots in the sequence from the spring varieties indicate identical bases with the sequences from the winter varieties. The three differences found between the 's' and 'w' haplotypes are indicated by bold letters. *Aci*I restriction sites are underlined. The location of the allele specific primer 361W-F is indicated in underlined italic letters.

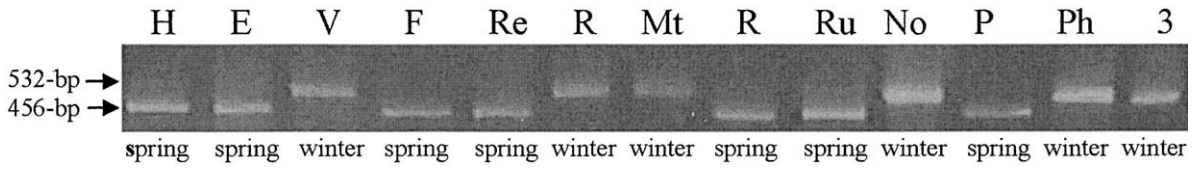

Fig. 3. PCR product amplified by *VRN-A1*-specific primers and digested with restriction enzyme *Aci*I. The growth habit of each variety is indicated below the photograph. The PCR product from the winter varieties is 76 bp larger than the PCR product from the spring varieties, which have an additional *Aci*I restriction site. The varieties used in this figure except HiLine are not part of Table 1: H-HiLine, E-Era, V-Vanguard, F-Federation, Re-Red Egyptian, R-Rocky, Mt-MTW94410, R-Reeder, Ru-Russ, No-Norstar, P-Pilot, Ph-Pronghorn, 3-PI372129.

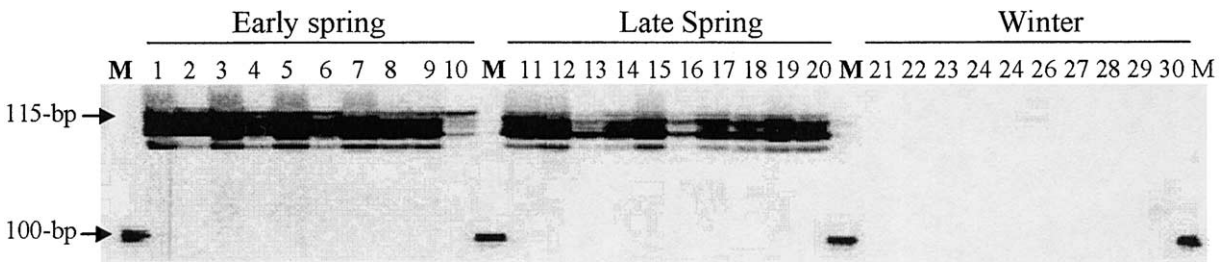

Fig. 4. PCR products from F_2 plants from the cross Newana × Redwin amplified by IRDye800-labeled primers 361SF and VA1-R and run on a Licor DNA Analyzer. Products were digested with restriction enzyme *Aci*I to confirm that they originated in the A genome. Note that the specific primers for the 's' allele amplified a product only from the 20 spring plants (1–20). The band is absent in the 10 winter individuals (21–30). Digestion with *Aci*I produced the expected band of 116 bp, confirming the presence of a different 's'-specific polymorphism in the PCR products. *M* indicates the molecular weight marker.

show the expected association between the dominant *VRN-A1* allele and the corresponding marker, indicating that these mutations are probably not directly responsible for the differences in growth habit. The two 1-bp indels are located within introns 4 and 6, and it is unlikely that they would affect the regulation of the *VRN-A1* transcription. The single SNP that differentiates the two haplotypes is located within the seventh exon and generates an amino acid difference, a valine in the w haplotype and an alanine in the s haplotype. However, an alanine amino acid is also present at the same position in the *VRN-1* sequences from winter *T. monococcum* accession G1777 (Yan et al., 2003) and winter *T. tauschii* accession AL8/78-2-2 used to construct the D genome BAC library (GenBank AY466446). These results suggest that the valine to alanine amino acid difference is not sufficient to explain the change between winter and spring growth habit. However, it would be interesting to investigate if this amino acid difference in the VRN-A1 protein in hexaploid wheat has any effect on flowering time.

In diploid wheat *T. monococcum*, no amino acid differences in the VRN-A1 proteins were found between accessions with spring and winter growth habit. The differences in growth habit were associated with deletions in the promoter regions (Yan et al., 2003). A more detailed study of the allelic variation in the promoter region of the *VRN-A1* gene in hexaploid wheat will be necessary to establish the relationship between the haplotypes found in this study and the differences in the regulatory regions of the *VRN-A1* gene.

Although the polymorphisms discovered in this study are probably not the direct cause of the differences between spring and winter growth habit, they were associated with growth habit in a wide range of germplasm (Table 1). The three spring accessions with the w haplotype were shown to carry a recessive *vrn-A1* allele and alleles for spring growth habit at different *VRN-1* loci, and so were not real exceptions (Table 1). The only two real exceptions were Chinese Spring and Triple Dirk C, which have the recessive *vrn-A1* allele but showed the three mutations characteristic of the s haplotype. A simple explanation for these observations is that the separation of the s and w haplotypes occurred before the mutation that originated the change in growth habit, and that those mutations occurred in *VRN-A1* genes with the s haplotype. Thus far, all springs have the s haplotype and most accessions with the recessive *vrn-A1* allele winters have the w haplotype. The close association between haplotypes and growth habit support the conclusions of Yan et al. (2003) that the wheat *AP1* gene is the vernalization gene *VRN-1*.

Breeding Applications

The results from the Newanan × Redwin population indicates the feasibility of using these markers to screen populations segregating for the *VRN-A1* gene. Even though these markers are based on polymorphisms that are not the cause of the differences in growth habit, the probability of a recombination between this marker and a different part of the *VRN-1* gene is extremely small. Yan et al. (2003) did not find a single recombinant within the *VRN-A1* gene in a segregating population of 6190 gametes. The large regions of repetitive elements flanking the *VRN-A1* gene (at least 50 kb in one side and 165 kb on the other) might contribute to the low recombination frequency observed in this region. Therefore, for this type of application these markers would probably be as reliable as a perfect marker.

The CAPS marker is the better option for applications that require a codominant marker. For example, in a marker assisted backcrossing programs to convert a spring line into a winter growth habit, the CAPS marker can be used to differentiate the *VRN-A1* homozygous and heterozygous spring plants. The CAPS marker also can be used to select homozygous plants for the spring haplotype in an F_2 segregating population, to fix the spring growth habit. However, if the breeding objective were to enrich very large segregating populations for one of the alleles, the allele specific primers would probably be a cheaper option. These markers do not require a restriction enzyme digestion after the PCR, minimizing time and cost.

These molecular markers also could be useful in characterizing spring lines of unknown genotype. As an example of this application, the CAPS marker was used to characterize seven spring accessions (Fig. 3). All these accessions had the s haplotype suggesting that they carry the dominant spring allele for the *VRN-A1* locus. The five winter lines included as controls showed the expected w haplotype (Fig. 3).

The CAPS marker also can be used to screen large germplasm collections and identify spring accessions

carrying alleles for spring growth habit different from *VRN-A1*. The identification of lines Egypt NA 101, Gular, and S-1 carrying spring alleles different from *VRN-A1* is a good example of this type of application. The quantification of the frequencies of the different spring alleles in different regions of the world might provide an insight on the adaptive value of the different *VRN-1* alleles to different environments.

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