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Detection of SNPs in the *VRN-A1* gene of common wheat (*Triticum aestivum* L.) by a modified Ecotilling method using agarose gel electrophoresis

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Abstract

Vernalization is one of the most important traits for the adaptability of wheat; detecting variation in genes regulating the response of wheat to vernalization is of importance for classifying winter phenotypes. Ecotilling is a high throughput reverse genetics approach for detecting single nucleotide polymorphisms (SNPs) in the genes of a natural population. To simplify methodology and decrease costs, a modified Ecotilling method based on agarose gel electrophoresis was used to discover the occurrence of SNPs in the wheat vernalization gene *VRN-A1* in a natural population of 106 accessions of hexaploid wheat varieties and their association with the winter/spring phenotype. Based on the SNPs detected, these accessions fell into three haplotypes, namely, ´A´, ´B´ and ´C´, each with five, six, and three variations, respectively. The winter phenotype was mainly determined by haplotype ´A´ and ´B´, while the spring phenotype was mainly determined by haplotype ´C´. After sequence confirmation, three SNPs (´A/-´, in the fourth intron; ´-/A´, in the sixth intron; ´T/C´, in the seventh exon) were associated with wheat vernalization and another three mutations (´G/T´ in the second intron; ´T/C´ in the fourth exon; ´CA/--´, in the fourth intron) had no identified effect. Therefore, Ecotilling with detection of DNA fragments by agarose gel electrophoresis can be used as an efficient method for detecting SNPs in genes of polyploidy materials, such as wheat.

Keywords: Agarose gel electrophoresis; Ecotilling; SNP detection; Vernalization; *VRN-A1* gene; Wheat.
Abbreviations: CELI- a single-strand specific endonuclease extracted from celery; EB- Ethidium bromide; Ecotilling- A high throughput reverse genetics approach for detecting single nucleotide polymorphisms in the genes of a natural population; N5AT5D- Chinese Spring nulli-tetrasomic line without the 5A chromosome but with two pairs of the 5D chromosomes; N5BT5D- Chinese Spring nulli-tetrasomic line without the 5B chromosome but with two pairs of 5D chromosomes; N5DT5B- Chinese Spring nulli-tetrasomic line without the 5D chromosome but with two pairs of 5B chromosomes; SNPs- Single Nucleotide Polymorphisms; TAE- Tris, Acetic acid and EDTA buffer for agarose gel electrophoresis; Tilling- Targeting induced local lesions in genomes; *VRN-A1*- The vernalization gene *VRN1* in the 5A chromosome of hexaploid wheat.

Introduction

Vernalization, the requirement of an exposure to low temperatures to hasten flowering, is an essential adaptation of plants to cold winters. In hexaploid wheat, the requirement for vernalization is mainly regulated by the vernalization gene *VRN1*, which has three homologous alleles, *VRN-A1*, *VRN-B1*, and *VRN-D1* which locate to the long arm of chromosomes 5A, 5B, and 5D, respectively (Law et al., 1976; Iwaki et al., 2002). The *VRN-A1*, *VRN-B1*, and *VRN-D1* genes are dominant for spring growth habit and epistatic to the alleles for winter growth habit. Therefore, winter cultivars are homozygous for the recessive alleles at the three *VRN-1* loci (Stelmakh 1987; Yan et al., 2004). However, the effects of these three genes on vernalization were different, with *VRN-A1* having the strongest epistatic effect on the other two genes (Pugsley, 1971). Sherman et al. (2004) identified three nucleotide changes that differentiated the dominant (spring haplotype) and recessive (winter haplotype) *VRN-A1* alleles. Yan et al. (2004) characterized the allelic variation at the *VRN-1* promoter region in polyploidy wheat and found that size polymorphisms in the promoter region were associated with the dominant and recessive *VRN-A1* alleles. Moreover, Fu et al. (2005) reported that the large deletions within the first intron in *VRN-1* were also associated with spring growth habit in wheat. In this research, we looked for the presence of single nucleotide polymorphisms (SNPs) in the key gene *VRN-A1* in 108 accessions from natural populations of wheat from China by Ecotilling and the correlation between the SNP haplotype and the winter and spring phenotypes among the accessions was investigated. Targeting induced local lesions in genomes (Tilling) is a reverse genetics method for detecting point mutations. In this method, a single-strand specific endonuclease extracted from celery (CELI) is used to shear DNA where there are mismatched bases in the DNA heteroduplexes of PCR products. A common method for detecting the presence of an SNP is to use primers labeled with two different fluorescence dyes (McCallum et al., 2000; Till et al., 2003). The high throughput and sensitivity of this

technique make it very valuable for screening SNPs in large-scale artificially induced mutant populations. Tilling techniques have already been successfully used to identify the point mutations in genes of interest in mutant populations of important species such as Arabidopsis (*Arabidopsis thaliana* L.) (Alonso et al., 2006), corn (*Zea mays* L.) (Till et al., 2004), rice (*Oryza sativa* L.) (Suzuki et al., 2008), and wheat (*Triticum aestivum* L.) (Slade et al., 2005; Parry et al., 2009). Ecotilling uses the same principle as Tilling to detect SNPs in genes in a natural population and has already been applied in various researches on species of Arabidopsis (Comai et al., 2004), rice (*Oryza sativa* L.) (zhang et al., 2008), barley (*Hordeum Vulgare* L.) (Mejlhede et al., 2006) and cabbage (*Brassica rapa* L.) (Wu et al., 2005). Specific primers labeled with a fluorescent dye, and subsequent detection of the labeled DNA fragments by fluorescence imaging restricts the use of the more common method of Tilling/Ecotilling because of complexity and cost. Several studies have proposed that the first amplification can be accomplished by adding specific primers with universal M13 adaptor sequences at their 5' end, subsequently accomplishing the second amplification using its products together with the IRD-labeled M13 primers such that the amplified fragments possess fluorescence which can facilitate detection (Wu et al., 2005). By applying this method, the cost for synthesis of a large amount of specific fluorescence-labeled primers can be saved. However, it is difficult to optimize the ratio between two primer pairs due to unstable amplification. Since the optimization of amplification conditions is relatively complicated (Wu et al., 2005), depending on the fluorescence detection system, it is still not widely applied. The costly and complex procedures can be replaced by a technique based simply on agarose gel electrophoresis (Raghavan et al., 2007) or on polyacrylamide gel electrophoresis (Kadaru et al., 2006) and general staining of the separated DNA fragments. This retains the advantages of high throughput and high sensitivity and introduces the advantage of an increase in size of the part of genes in which SNPs can be detected. For example, through agarose gel electrophoresis, SNPs in gene fragments with a length over 2 kb can be detected (Raghavan et al., 2007). Although this method is not better than the fluorescence detection system in terms of sensitivity and intuitive judgment of the results, it has prospects for wider application and has already been adopted for some studies (Zhang et al., 2008; Raghavan et al., 2007).

Materials and methods

Plant materials

A natural population of 106 common bread wheat (AABBDD) accessions from China, including 66 cultivars, 33 advanced lines, and 7 landraces, as well as two durum wheat (AABB) lines, GD-1 and GD-2, was used in this study. The materials were grown at the No.1 Farm of Northwest A&F University, Yangling, Shaanxi in October 16 during the 2007-2009 growing seasons. Their winter phenotypes were recorded as their performance during winter in the field and referred from literature. The name, origin, and winter phenotypes of the accessions are listed in the supplementary table. The most known wheat cultivar, Chinese Spring, was used as control due to its importance and prevalence in the study of wheat genetics.

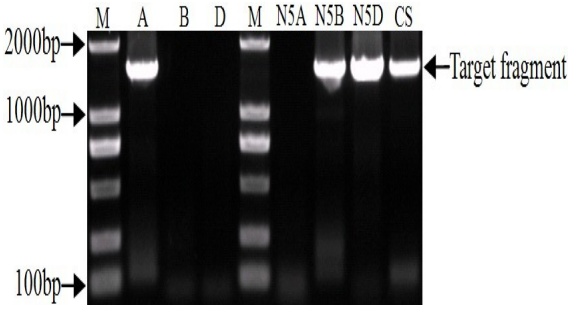

Fig 1. The amplification of primer VRN-A1 in *T. urartu* (AA), *T. speltoids* (BB), *Ae. tauschii* (DD) and relevant nulli-tetrasomic lines N5AT5D, N5BT5D and N5DT5B of Chinese Spring. A: *T. urartu*; B: *T. speltoids*; D: *Ae. tauschii*; N5A: Chinese Spring nulli-tetrasomic line N5AT5D without the *VRN-A1* locus; N5B: Chinese Spring nulli-tetrasomic line N5BT5D with the *VRN-A1* locus; N5D: Chinese Spring nulli-tetrasomic line N5DT5B with the *VRN-A1* locus; CS: Chinese Spring; M: DL2000 molecular weight standards.

Extraction of crude CELI *from celery and detection of its activity*

Extraction of crude CELI was carried out following the method described by Yang et al. (2000) and Oleykowski et al. (1998). The endonuclease activity and stability of the CELI preparations were determined by analyzing the heteroduplexes formed by PCR fragments from a mix of a pair of plasmids containing known mutations using a Li-Cor 4200 genetic analyzer (Li-Cor, Lincoln, Nebraska USA) with fluorescence-labeled M13 primers (Till et al., 2006).

Primer design and specificity identification

Wheat (*Triticum aestivum* L.) is an allohexaploid species with three closely related genomes. Most wheat genes are present as three similar sequences of homoeologous copies. To design genome specific primers, sequences for *VRN-1* (*VRN-A1*,*VRN-B1* and *VRN-D1*) of wheat were downloaded from GenBank and aligned with each other, then PCR primers specific for *VRN-A1* were designed using the web-based tool CODDLE (http://proweb.org/coddle) (McCallum et al., 2000) based on the genome sequence of *VRN-A1* gene in wheat material IL369 (GenBank: AY747599) as follows: VRN-A1F: 5'-gga, gat, gaa, acc, caa, aag, cca, aag, agc- 3' and VRN-A1R: 5'-gcc, ggt, gca, act, tgt, tac, cct, cta, ctg-3'. PCR amplification was performed using the genomic DNA of Chinese Spring as the template. The amplified product was cloned to the pMD-18-T vector (TaKaRa, Dalian) and sequenced to determine its specificity. Additionally, PCR was also performed using diploid wheat materials of the *T. urartu* (AA), *T. speltoids* (BB) and *Ae. tauschii* (DD) and the nulli-tetrasomic lines N5AT5D, N5BT5D and N5DT5B of Chinese Spring to verify the primer's genomic specificity.

Genomic DNA isolation and preparation of DNA pooling

The genomic DNA from wheat leaves was isolated using the CTAB method (Aldrich, 1993) and diluted to 80 ng/μL. In a preliminary experiment, the genomic DNA from three to five wheat accessions was pooled and used as template for PCR amplification. The heteroduplexes formed were subjected to

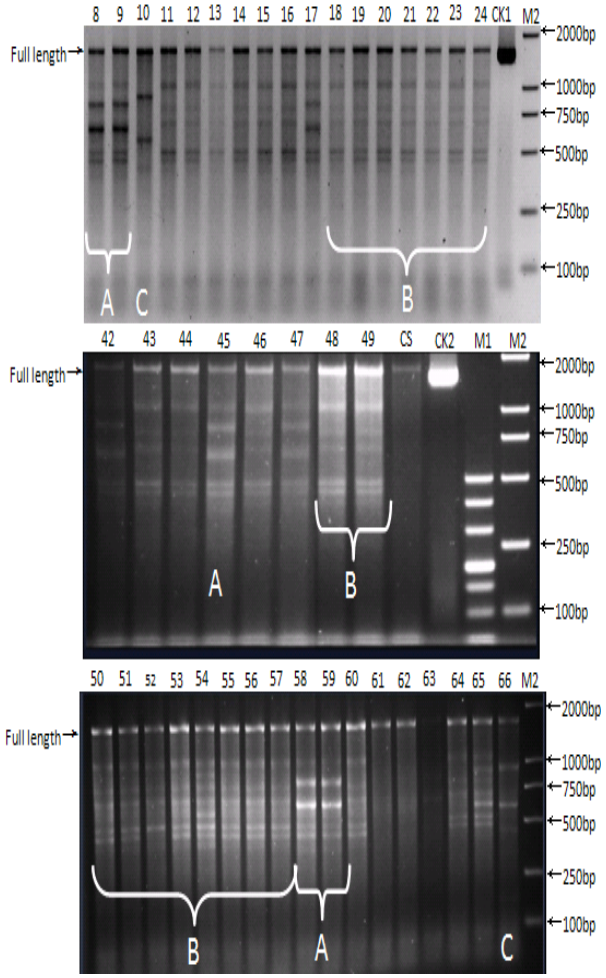

Fig 2. Ecotilling band patterns of *VRN-A1* gene in pools of some wheat accessions with Chinese Spring as the reference by agarose gel electrophoresis. M1 and M2 are molecular weight standards DL500 and DL2000, respectively; ´CS´ is Chinese spring as the reference; CK1 and CK2 is the non-CELI digested PCR products of DNA pools of accession 24 and 49, respectively; 8~24, 42~49, and 50~66 are the code number of DNA pools of the corresponding accessions; the full length of the target fragment is 1511 bp. The band patterns of the CELI digested PCR products for the haplotype ´A´, ´B´, and ´C´ is indicated by A, B and C, respectively.

the CELI digestion and the digested fragments were separated by agarose gel electrophoresis. Because there were too many bands for easy analysis, it was decided that, for the present study, the DNA pool should be built with the genomic DNA of one test wheat accession and the control, Chinese Spring, only at a ratio of 1:1 for PCR amplification.

PCR amplification and heteroduplex formation

The PCR reaction was conducted in 20 µL containing 13.4 µL deionized water, 2 µL 10×Ex Taq Buffer (TaKaRa), 1.4 µL dNTPs (2.5 mM each dNTP), 0.5 µL each of 10 µM forward and reverse primer, 2 µL DNA template (pool of the test accession and the control at ratio of 1:1), and 0.2 µL of Ex Taq DNA polymerase (5 U/µL, TaKaRa). The PCR profile was as follows: 95ºC for 5 min; followed by 8 cycles of touchdown at 94ºC for 20 s, an annealing step starting at 75ºC for 20 s and decreasing by 1ºC per cycle, 72ºC for 90 s; then 40 cycles of 94ºC for 20 s, 67ºC for 20 s, 72ºC for 90 s; and finally elongation at 72ºC for 5 min; a denaturing and re-annealing step is included at the end of the PCR reaction (99ºC for 10 min, followed by 70 cycles of 70ºC for 20 s decreasing by 0.3ºC per cycle) to allow the formation of heteroduplexes if SNPs are present in the pool.

CELI *digestion of the heteroduplexes and agarose gel electrophoresis detection*

Heteroduplexes (20 µL) formed after PCR amplification, denaturation and annealing, were mixed with 20 µL CELI mixture, containing 6 µL CELI Buffer (10 mM Hepes, pH 7.5, 10 mM $MgSO_4$, 10 mM KCL, 0.002% Triton X-100, and 0.2 µg/mL BSA); 2.4 µL CELI preparation, and 11.6 µL ultrapure water. The digestion was then commenced at 45ºC for 45 min. Subsequently, 4 µL 10×Loading Buffer was added to stop the reaction. The digested products were separated on 2% agarose gels with 1×TAE buffer at 110 V for 50 min, stained in an ethidium bromide (EB) buffer for 15 min, and then visualized by Gel Doc XR (BioRad Laboratories, Inc.).

Genotyping and verification by sequencing

The differences in size of the digested fragments from various DNA pools was assessed on the agarose gel electrophorograms, and the accessions with the same band patterns were regarded as the same genotype for haplotype grouping. Subsequently, two accessions grouped in each of the haplotypes were chosen randomly and used for sequencing analysis. The target region of wheat *VRN-A1* for each of the accessions chosen was amplified and cloned into the T-vector. Three positive clones for each accession were sent for sequencing. Sequence alignment and comparison were conducted with BioEdit (Hall, 1999), and the consistency between sequence and digested fragment were checked. The sequences obtained were used to compare and confirm the conclusions from Ecotilling. Blast analysis was performed online in the NCBI Web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Accessions with the same band patterns were classified into the same group, and the winter phenotype of each accession in this group was analyzed to see if the accessions from the same group had the same or similar phenotype. Then the SNPs detected in the representative accessions were used to discover the association between SNPs and vernalization phenotype.

Results

Validation of primer specificity

PCR amplifications with primer VRN-A1 were carried out and optimized using the genomic DNA of Chinese Spring as template. A single target band was obtained. The band was recovered from agarose gel, purified and cloned into the pMD-18-T vector (TaKaRa, Dalian). Six positive clones were picked randomly for sequencing. It was suggested by the sequence alignment of the 6 clones that the target fragment amplified was consistent and that there was no non-specific amplification. In addition, the primer could amplify the right product in the wheat A genome donor *T. urartu* and the nulli-tetrasomic lines (N5BT5D and N5DT5B) of Chinese Spring, while there was no product amplified for

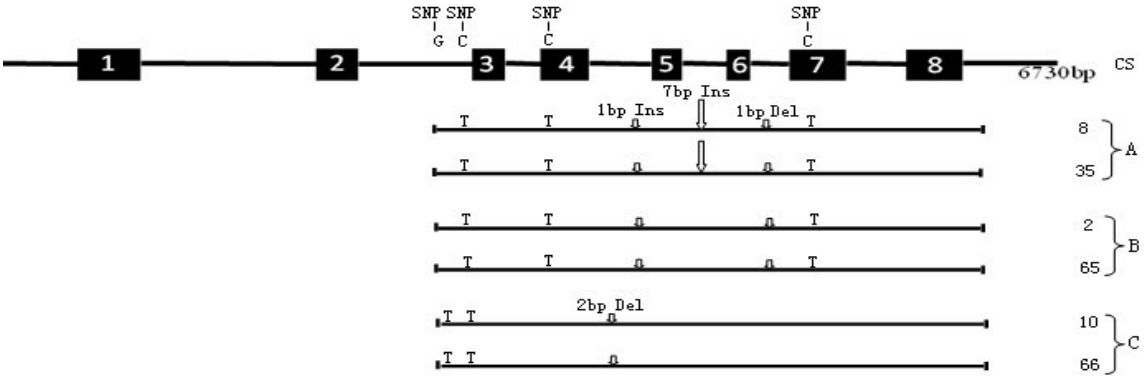

Fig 3. Sequence comparison in the amplified *VRN-A1* gene region between haplotypes ´A´, ´B´, and ´C´ with Chinese Spring, respectively. ´CS´ represents the complete coding region of *VRN–A1* gene in Chinese Spring (CS). The introns and exons were indicated by black lines and black boxes, respectively. Sequences from the representive accessions of each haplotype were aligned with ´CS´ (from 4965 bp to 6475 bp with full length of 1511 bp). ´A´ shows the target sequences of 8 and 35 of haplotype ´A´, which had 6 mutations with ´CS´.´B´ shows the target sequences of 2 and 65 of haplotype ´B´, which had 5 mutations with ´CS´. There was only one mutation different between haplotype ´B´ and ´A´. ´C´ shows the target sequences of 10 and 66 of haplotype ´C´, which had 3 mutations with ´CS´ and all of them were in introns.

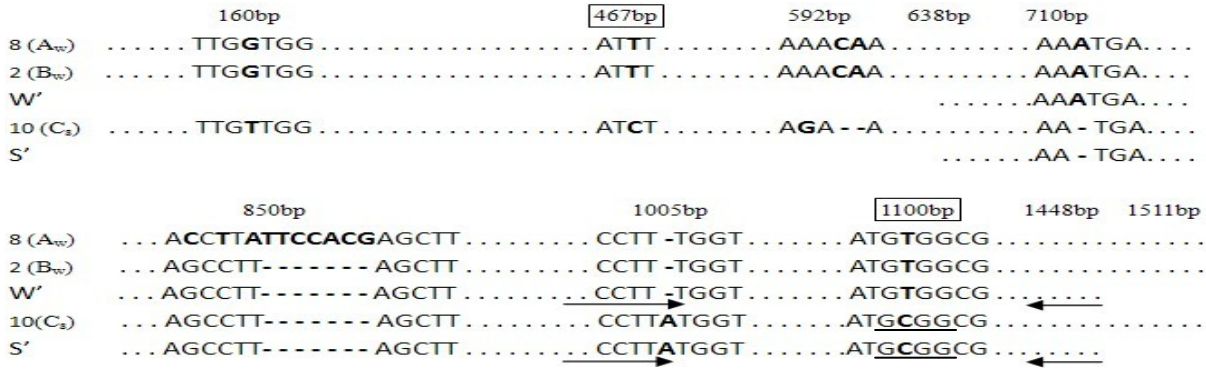

Fig 4. Sequence comparison for target region of the *VRN-A1* gene between winter and spring genotypes. 8(Aw) is haplotype ´A´ accession 8 with winter genotype; 2(Bw) is haplotype ´B´ accession 2 with winter genotype; 10(Cs) is haplotype ´C´ accession 10 with spring genotype. The seven mutations found between the winter and spring genotypes are indicated by bold letters. W' and S' are from Sherman et al. (2004) with full length of 810 bp for winter and spring genotypes, respectively; the locations of the STS primer sites are indicated by arrows under the corresponding letters; the *Aci*I restriction site for the CAPS marker is underlined.

either the wheat B genome donor *T. speltoids* or wheat D genome donor *Ae. tauschii*, and the nulli-tetrasomic line (N5AT5D) of Chinese Spring as well (Fig. 1). These results suggested that the primer designed was of favourable specificity for amplifying the *VRN-A1* fragment and could be used for the experiments.

Detection of digested products by agarose gel and genotyping

Band patterns for the *VRN-A1* gene obtained by Ecotilling for some wheat accessions pooled with Chinese Spring are shown in Figure 2. Differences of band patterns among the digested products of different pools are clearly observed by agarose gel electrophoresis. As many as eight digestion fragments were detected in some pools, by which more than two digestion fragments from one amplified fragment could be detected on agarose gels stained by ethidium bromide (EB). Such fragments could not be detected by the Li-Cor system since no fluorescence is present in those fragments if both ends of the fragment are cut off. Therefore, more digestion fragments could be detected by agarose gel electrophoresis than by the Li-Cor system. Sequence variations in the *VRN-A1* gene between various wheat accessions pooled with Chinese Spring were shown by analyzing the band patterns of CELI digested fragments of heteroduplexes formed in the PCR products of the *VRN-A1* gene in the corresponding pools of genomic DNA. Figure 2 shows that the band patterns from various pools of wheat accessions with Chinese Spring were quite different. Only the major differences in band patterns were considered for haplotype classification; minor differences in band patterns were disregarded. Based on similarities and differences in the band patterns of different pools, the 108 wheat accessions were divided into three groups, namely, haplotype ´A´, ´B´ and ´C´. Haplotype ´A´ was characterized by two specific bands of around 660 bp and 840 bp, with 27 wheat accessions classified into the group such as Shijiazhuang 8, Hanyou 98, Xinong 811, Changwu 58 and Jiufeng 22. Haplotype ´B´ included two specific bands at around 700 bp and 810 bp, with 68 wheat accessions classified into this group such as Shan 229, Xiaoyan 6, 151-3,

Table 1. Polymorphisms in the target region of *VRN-A1* gene between Chinese Spring and the three haplotypes.

Haplotype	locations	Intron/Exon	Nucleotide change	Amino acid change
A	5231[a](267[b])	Intron 2	C/T	-
	5431(467)	Exon 4	C/T	L/F
	5673(710)	Intron 4	-/A	-
	5811(850)	Intron 5	-/TTCCACG	-
	5962(1005)	Intron 6	A/-	-
	6057(1100)	Exon 7	C/T	A/V
B	5231(267)	Intron 2	C/T	
	5431(467)	Exon 4	C/T	L/F
	5673(710)	Intron 4	-/A	-
	5962(1005)	Intron 6	A/-	-
	6057(1100)	Exon 7	C/T	A/V
C	5124(160)	Intron 2	G/T	-
	5231(267)	Intron 2	C/T	-
	5558 (592)	Intron 4	CA/-	-

5231[a] indicates the position 5231 bp from the first base ´G´ of the *VRN-A1*gene (AY747599); 267[b] indicates the position 267 bp from the first base of the target sequence amplified in this study.

Jing 411 and Qinnong 712. While haplotype ´C´ included two specific bands at around 590 bp and 920 bp, with 13 wheat accessions belonging to this group such as Banong 1 and Xiaobingmai 32.

Verification of digested products detection by sequencing

To verify the reliability and accuracy of detection in the SNP haplotype of *VRN-A1* in wheat accessions by Ecotilling, two accessions in each haplotype were chosen at random and their amplified fragments of the *VRN-A1* gene were sequenced. The sequences obtained indicated consistent target fragment sequences in the two accessions of the same haplotype group, indicating that a relatively accurate SNP genotyping could be achieved in the target genes via the Ecotilling technique based on agarose gel electrophoresis. Alignment of the sequences (Fig. 3) showed that multiple SNPs existed in the target fragment of *VRN-A1* gene between the test wheat accessions and Chinese Spring, including conversion, transversion, and deletion of bases, as well as insertion of a small fragment. Herein, transversion from ´C´ to ´T´ was the most frequent event, accounting for 50% of the total mutations. The sequence alignment of each haplotype with Chinese Spring shows six mutations in haplotype ´A´, five mutations in haplotype ´B´, and three mutations in haplotype ´C´ (Table 1). In this study, neighbor continuous mutation sites were regarded as one mutation. Thus, it could be predicted that there would be 27 digestion fragments in haplotype ´A´, 20 digestion fragments in haplotype ´B´, and 9 digestion fragments in haplotype ´C´. In fact, not all of the expected fragments could be detected by agarose gel electrophoresis. For instance, only eight clear bands could be detected in haplotype ´B´. This may result from the lower detection sensitivity of agarose gel electrophoresis or the insufficient quantities of small fragments because of cleavage at multiple mismatched bases in the target gene by CELI. Haplotype grouping could, nonetheless, still be relatively and accurately performed since obvious fragment patterns were present due to mutation differences at the key sites. While checking the correlation between the haplotype detected by Ecotilling and the winter phenotypes of those wheat accessions, it was observed that all the wheat accessions with haplotype ´A´ and ´B´ of the *VRN-A1* gene were of winter (48% of haplotype ´A´ and 54% of haplotype ´B´) or facultative (52% of haplotype ´A´ and 46% of haplotype ´B´) types, while all the wheat accessions with haplotype ´C´ of the *VRN-A1* gene were of the spring type (see supplementary Table). This suggested that the haplotype based on band patterns of Ecotilling analysis with agarose gel detection could well reflect the variation of the *VRN-A1* gene between winter and spring types. The result was consisted with that of Sherman et al. (2004), who compared the 810 bp segment of the *VRN-A1* gene between intron 4 and exon 8 of 77 accessions and identified two main haplotypes (W' and S') that differed in two 1 bp indels and one single nucleotide polymorphism (Fig. 4). The two 1 bp indels (´A/-´; ´-/A´) were located within introns 4 and 6, the same as ours at 710 bp and 1005 bp of the target region; the single SNP (´T/C´) was like ours located within the seventh exon at 1100 bp (Fig. 4). They found that accessions with W' haplotype had the winter phenotype (except three accessions, where the spring phenotype was determined by a dominant allele at one of the other vernalization genes), while accessions with S' haplotype were spring phenotype (except Triple Dirk C). In our research, more than three mutations in the target region were found between the winter genotype and spring genotype. Three mutations are found within introns 2 and 4, and in exon 4, which were outside the region amplified by Sherman; the three of the other 4 mutations found within introns 4, 6, and exon 7 were the same as Sherman's key nucleotide changes. A new SNP mutation within intron 5 at 850 bp in haplotype ´A´ was the only SNP between haplotype ´A´ and haplotype ´B´, and it was not consistently associated with the winter phenotype, so we suggested that it was not the key SNP determining wheat vernalization. The results indicate that agarose Ecotilling is feasible for discovering allelic variation. Although not all the expected bands for the different wheat genotypes were detected by Ecotilling analysis based on agarose gel electrophoresis, the differences detected in band patterns were sufficient to reflect the major differences in the target between different genotypes. The method is shown to be a fast, convenient, and effective method for detecting SNPs in genes of polyploidy species, such as wheat.

Discussion

Allelic variation in wheat VRN-A1 gene and its relation with wheat phenotype

Sherman et al. (2004) identified three nucleotide changes between the dominant *Vrn-A1* and recessive *vrn-A1* alleles. Then two of the differences were exploited to develop a CAPS marker (cleavage amplification polymorphic sequence) and a STS marker (sequence-tagged site primers),

respectively (Fig. 4). Zhang et al. (2006) compared the STS marker and CAPS marker by testing 22 Chinese wheat cultivars; the testing results of STS marker were completely consistent with those of CAPS marker, suggesting that the STS marker is more efficient and reliable for identifying *Vrn-A1* gene in wheat breeding program. The three mutations found by Sherman et al. (2004) are probably not the direct cause of the differences between spring and winter growth habit, though they were associated with winter phenotype in a wide range of germplasm. In this study, the three mutations were also detected by the band patterns of the Ecotilling analysis and provide a good reference in distinguish winter and spring phenotypes. Additionally, a longer gene fragment than that of Sherman et al. (2004) was amplified and another three stable mutations between the winter and spring genotype (160 bp, 470 bp, 592 bp, Fig. 4) were found. These can be developed to STS markers in future studies. However, other studies proved that the differences in the promoter region or the first intron of the *VRN-A1* gene were also the key factors differing between winter and spring genotype (Yan et al., 2004; Fu et al., 2005). Based on these results, we hypothesize that the three regulatory sequences are all required for the vernalization response. The dominant and recessive variations of vernalization genes among different wheat cultivars could be detected by the methods mentioned above. In this study, SNPs of *VRN-A1* gene in wheat accessions were detected and analyzed using a modified Ecotilling method based on agarose gel electrophoresis, by which base differences among different genotypes could be revealed quite accurately. In addition, the haplotypes revealed by band patterns were well correlated to the winter phenotypes of wheat accessions, which provide a new method for allele detection of the *VRN-A1* gene and other important genes in wheat.

Application of the modified Ecotilling method and its drawbacks

Variations in a single nucleotide and insertion or deletion of a small fragment of sequence are the most common causes of genetic diversity in a natural population (Wright et al., 2005; Kumar et al., 2009). It has been demonstrated that certain correlations exist between SNPs and many of the key characters of crops; even the phenotype of some characters was indicated by certain SNPs. For example, the wheat dwarf gene *Rht1* was the result of a single nucleotide mutation in the gene coding region such that the plant type changed from tall to short (Peng et al., 1999). Therefore, discovering new SNPs in important functional genes and identifying their relationship with ideal phenotypes have become critical approaches in the genetic improvement of crops. With rapid developments in plant genome sequencing techniques, more genomic sequences for plants are becoming available, making it possible to employ a reverse genetics method for discovering gene functions. Tilling and Ecotilling techniques have become important methods for discovering novel SNPs either in a mutated or a natural population. Traditional Tilling and Ecotilling techniques depend on fluorescence-labeled primers and expensive genetic analysis equipment, and are thus difficult to apply in most laboratories. In recent years, capillary electrophoresis (Suzuki et al., 2008), polyacrylamide gel electrophoresis (Kadaru et al., 2006; Uauy et al., 2009), and agarose gel electrophoresis (Raghavan et al., 2007; Dong et al., 2009) have been applied to separate digested products of the Tilling and Ecotilling system. An efficient modified Ecotilling method for discovering SNPs in wheat genes is described, which is optimized with respect to template DNA pooling, the PCR amplification reaction, CELI digestion and detection of digested products by agarose gel electrophoresis. The technique was used to detect SNPs in the wheat *VRN-A1* gene in a natural population. In the Tilling analysis detection by the Li-Cor genetic analyzer, the usual ratio for DNA pooling was 1:7 (McCallum et al., 2000). For Ecotilling, the number of test materials in each pool needs to be reduced since variations exist in natural population at higher frequency than those in artificially mutated populations, especially in polyploidy species such as wheat with a large genome. DNA pooling in this study was conducted by mixing the genomic DNA of the test material and the control in a 1:1 ratio. Additionally, the amount of template used in PCR was increased to 160 ng DNA in the 20 μL reaction, thus achieving favorable results although this was not consistent with the experience of Raghavan et al. (2007), who adopted 2.5 ng DNA a 10 μL reaction for Ecotilling analysis in rice. Possible reasons for this variance may be the size difference of genomes between hexaploid wheat and rice, and the CELI activity. Furthermore, for detection with agarose gel, the purification and concentration of digestion products before electrophoresis were omitted compared with the detection by fluorescence. Therefore, to achieve better results for Ecotilling analysis in wheat by agarose gel detection, it was important to obtain sufficient digested products by using more DNA in the reaction mixtures. Tilling and Ecotilling techniques using the Li-Cor genetic analyzer as the detection platform based on the laser scanning imaging, were suitable for detecting SNP in 1200 bp~1500 bp target regions. For agarose gel electrophoresis, regions with a length of 2 kb~3 kb could be targeted (Raghavan et al., 2007), enabling effective detection of SNPs in genes with longer sequences. Heteroduplexes could be formed by the PCR products of the test material itself while non-specific amplification occurred, further influencing the accuracy of Ecotilling analysis. Therefore, designing and selecting the genome specific primers and using the appropriate PCR conditions were crucial to obtain the right PCR product. Better methods for designing and validating genome specific primers in polyploidy species include the sequencing of the diploid donors of the different wheat genomes, then to design and select the best primers (Uauy et al., 2009). The specificity of primers could also be tested by CELI digestion of the PCR products of the control material itself, which may accelerate the progress of primer screening. 2%~2.5% agarose gels were selected for electrophoresis of digested products, because fragments with different sizes were then well separated. The volume of digests loaded was maintained at about 12 μL for each well so that the staining and imaging of small fragments could be guaranteed. It was suggested by Yang et al. (2004) that haplotype grouping for test materials could be decided from band patterns given by fragments released by CELI. In this study, the accessions were classified into three groups according to their band patterns shown by agarose gel electrophoresis. Some fragments expected from the results of sequencing were not detected by agarose gel electrophoresis, but genotyping of materials were not affected. At present, no method for SNP identification can determine the number, position and type of mutation directly, except for those in combination with the sequencing information of the PCR-amplified fragments. For most research, it is not necessary to completely determine the number and position of SNPs before sequencing. The presence or absence of SNPs existing in the DNA pool was effectively shown by Tilling/Ecotilling analysis with agarose

gel electrophoresis; Therefore, Ecotilling analysis based on agarose gel electrophoresis possesses many advantages over the Li-Cor system, including time-saving, labor-saving, easy operation and low cost. Here it was shown to be convenient for application to SNP detection, and possesses huge potential value, especially in polyploidy crops.

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Supplementary Table. The name, origin, winter phenotype of wheat accessions used in this study and their haplotype of *VRN-A1* gene.

ID	Name	Origin[a]	Cultivar type	Winter hardiness	Haplotype of VRN-A1
1	Lingfeng 615	W	Cultivar	Winter	B
2	Yunhan 20403	F	Advanced line	Winter	B
3	Linhan 51329	F	Advanced line	Facultative	B
4	Linhan 536	F	Advanced line	Winter	B
5	Yunhan 22-33	F	Cultivar	Winter	B
6	Luohan 2	F	Cultivar	Facultative	A
7	Luohan 6	F	Cultivar	Facultative	B
8	Shijiazhuang 8	F	Cultivar	Facultative	A
9	Hanyou 98	F	Cultivar	Facultative	A
10	Banong 1	F	Cultivar	Spring	C
11	NC223	W	Advanced line	Winter	B
12	Zhonghan110	W	Cultivar	Winter	B
13	Nongdayoumai2	F	Cultivar	Facultative	B
14	Changwu134	W	Cultivar	Winter	B
15	Jinmai47	W	Cultivar	Winter	B
16	Changwu 117	W	Cultivar	Winter	B
17	Changwu 863	W	Cultivar	Winter	A
18	Changwu 135	W	Cultivar	Winter	B
19	Changwu 521-7	W	Cultivar	Winter	B
20	Shan229	F	Cultivar	Facultative	B
21	Xiaoyan6	F	Cultivar	Winter	B
22	Shanmai168	F	Cultivar	Facultative	B
23	Shanhan187	F	Cultivar	Facultative	B
24	Xinong52	F	Cultivar	Winter	B
25	Xinong 981	F	Cultivar	Winter	B
26	Xinong 129	F	Cultivar	Facultative	B
27	Shan872	F	Cultivar	Facultative	B
28	Pubing201	F	Cultivar	Winter	A
29	Xinong961	F	Cultivar	Winter	A
30	Xinong H-248	F	Cultivar	Facultative	B
31	Shan512	F	Cultivar	Facultative	B
32	Xiaoyan22-3	F	Cultivar	Winter	A
33	Pubing151	F	Cultivar	Facultative	B
34	Pubing143	F	Cultivar	Winter	A
35	Xinong389	F	Cultivar	Facultative	A
36	Liken2	F	Cultivar	Winter	B
37	Lantian10	W	Cultivar	Winter	B
38	Luohan3	F	Cultivar	Facultative	A
39	Cang6003	W	Cultivar	Winter	A
40	YanD 27	F	Cultivar	Facultative	B
41	8718	F	Advanced line	Facultative	A
42	98601	F	Advanced line	Facultative	A
43	9322	F	Advanced line	Facultative	B
44	93166	F	Advanced line	Facultative	B
45	Xinong 811	W	Cultivar	Winter	A
46	Ningaifeng 1	F	Cultivar	Winter	B
47	9812-7	F	Advanced line	Winter	A
48	151-3	F	Advanced line	Winter	B
49	Jing 411	W	Cultivar	Winter	B
50	Jinan 13	F	Cultivar	Facultative	B
51	Chang 6878	W	Cultivar	Winter	B
52	Xinong 4504	F	Advanced line	Facultative	B
53	Xinong 794	F	Cultivar	Winter	B
54	Xinong 8325	F	Cultivar	Facultative	B
55	Q451	F	Cultivar	Winter	B

ID	Name	Origin[a]	Cultivar type	Winter hardiness	Haplotype of VRN-A1
56	T304	F	Advanced line	Winter	B
57	Qinnong 712	F	Cultivar	Winter	B
58	Changwu 58	F	Cultivar	Facultative	A
59	Jiufeng 22	F	Cultivar	Winter	A
60	04-135	F	Advanced line	Facultative	B
61	9769-29	F	Advanced line	Facultative	B
62	Ligao 6	F	Cultivar	Winter	B
63	Heng95Guan26	F	Cultivar	Facultative	A
64	Tongmai 3	F	Cultivar	Winter	B
65	Hongmangmai 1	S	Landrace	Winter	B
66	Xiaobingmai 32	W	Cultivar	Spring	C
67	DingXi 35	S	Cultivar	Spring	C
68	Ningdong 1	W	Cultivar	Winter	B
69	XiFeng 20	W	Cultivar	Winter	B
70	Ningchun10	S	Cultivar	Spring	C
71	Ningchun 20	S	Cultivar	Spring	C
72	Ningchun 27	S	Cultivar	Spring	C
73	Ningchun 34	S	Cultivar	Spring	C
74	Hyg44	Canada	Advanced line	Spring	C
75	hyg44(awnless)	Canada	Advanced line	Spring	C
76	QW6285	Canada	Advanced line	Spring	C
77	9866	Canada	Advanced line	Spring	C
78	604	Canada	Advanced line	Spring	C
79	H29	F	Advanced line	Winter	B
80	ATTLA	F	Advanced line	Winter	B
81	8901	F	Advanced line	Facultative	A
82	M178-8	F	Advanced line	Facultative	B
83	RD-14-2	F	Advanced line	Facultative	B
84	WT33-2	F	Advanced line	Facultative	B
85	AF-111-1	F	Advanced line	Facultative	B
86	9030	F	Advanced line	Facultative	B
87	Changwu61	F	Cultivar	Facultative	A
88	Changwu 58-61	F	Cultivar	Facultative	A
89	Changwu 982-3	F	Cultivar	Facultative	B
90	Yimai1	F	Cultivar	Facultative	A
91	GD-1	Guizhou	Advanced line	Facultative	B
92	GD-2	Guizhou	Advanced line	Facultative	B
93	Hongmangmai2	F	Landrace	Winter	B
94	Caihuangmai	W	Landrace	Winter	A
95	Hongtumai	S	Landrace	Winter	B
96	Jinbaoyin	S	Landrace	Winter	A
97	Daoshimai	S	Landrace	Winter	A
98	Halaqi	S	Landrace	Winter	A
99	Xuanxi101	F	Landrace	Winter	B
100	Jing14673	W	Landrace	Winter	B
101	PI225232	W	Landrace	Winter	B
102	PI222681	W	Landrace	Winter	B
103	Ganchun14	S	Cultivar	Spring	C
104	Changwu0318	F	Advanced line	Facultative	A
105	Chang989738-1-7	F	Advanced line	Facultative	B
106	Chang 98(3)6-7-2-6	F	Advanced line	Facultative	B
107	Chang 99(2)45-10	F	Advanced line	Facultative	B
108	Chang 99(3)F6-2-2-5-8	F	Advanced line	Facultative	B

[a]Note: F, Huang Huai Facultative Wheat Zone; S, Northwestern Spring Wheat Zone; W, North China Winter Wheat Zone.