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The Design of DNA Markers for the *Vrn-D1* Locus of Soft Wheat

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Abstract—A technique for the detection of the *Vrn-D1* locus in the wheat genotype by means of PCR markers was developed. It can be used for certification of varieties or individual wheat genotypes. Three DNA markers for the *Vrn-D1* locus (SPBC850, SPBC657, and SPBC780) were constructed using the PCR variants ISSR, RAPD, and STS, respectively. The dominant allele *Vrn-D1* was identified in varieties of spring soft wheat, substituted and isogenic lines of gene pools of different varieties of winter soft wheat, and in hybrid F_1 and F_2 populations. For the most thorough analysis of the pedigree seed, we suggest using all the three markers for the detection of the *Vrn-D1* locus.

Key words: DNA markers SPBC850, SPBC657, and SPBC780; PCR; soft wheat; Vrn-D1 locus

INTRODUCTION

Molecular markers of the second generation that enable the control of the most important agricultural properties are now used in order to increase the selection efficiency. The *Vrn* genes (namely, *Vrn-A1, Vrn-B1,* and *Vrn-D1*) can be classified among such markers: they are responsible for the types and development rates of soft wheat. The major function of *Vrn* genes is the control of differences in the duration of the organogenesis stage II; under certain conditions, they also control the stages VI and VII [1]. The continuance of these organogenesis stages quantitatively affects the development of germ cells of vegetative and generative organs, and, hence, produces the secondary effect on the harvest components and final productivity [2]. The difficulties in the obtaining of markers to *Vrn* genes result from their high homology due to the common origin and similar localization on 5AL, 5BL, and 5DL chromosomes [3]. Only a limited number of molecular markers to *Vrn* genes have been developed so far; for the most part, these markers belong to the RFLP class [4]. However, the RFLP method is known to be expensive and time-consuming, which impedes its wide use. At the same time, the need in such markers for a large-scale use in selection programs is urgent, and the PCR analysis seems to be the most promising approach in this aspect.

The goal of this work was the design of markers to the *Vrn-D1* locus using such PCR variants as ISSR, RAPD, and STS. The use of several methods and various types of primers allows a more thorough analysis of genomic DNA to reveal the specific sites associated with the *Vrn* gene under study.

EXPERIMENTAL

The following wheat varieties were used as the starting material: a set of nearly locus-isogenic *Vrn-A1, Vrn-B1,* and *Vrn-D1* lines in the gene pools of four winter soft wheat varieties Mironovskaya 808, Odesskaya 16, Skorospelka 3b, and Triple Dirk; seven varieties of spring wheat of various ecological and geographical origin: Beacon (Kenya), Cocoraque F75 and Kentana 48 (Mexico), Santa Catalina (Argentina), Sonalica (India), Erythrospermum 841 (Central Asia), and Bledsol (United States); a late-maturing analogue of Novosibirskaya 67-ANK18A soft wheat variety (Western Siberia, a kind gift of S.F. Koval', Institute of Cytology and Genetics, Siberian Division of Russian Academy of Sciences); null/tetrasubstituted lines at the fifth chromosome of Chinese Spring variety: null5A/tetra5B (H5A/T5B), null5B/tetra5D (H5B/T5D), and null5D/tetra5A (H5D/T5A); as well as individual plants F_1 and F_2, which are the monogenic hybrids of the

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spring line with the dominant *Vrn3* gene and its recurrent parent, winter wheat variety Mironovskaya 808 (recessive at all the three *Vrn* loci). Hybridological analysis was carried out according to [5]. High-molecular mass DNA was isolated from five-day shoots and young leaves according to the procedure in [6]. Amplification was performed on a Tertsyk SM2 device (DNA Technologies, Moscow) using the following regimes: DNA denaturation at 93°C for 1 min; elongation at 72°C for 40 s (final elongation for 3 min); and annealing with arbitrary primers at 46°C for 1 min, with ISSR primers at 58°C for 30 s, and at the STS analysis at 60°C for 30 s. The reaction mixture (20 μl) contained the buffer [50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.2 μmol of each dNTP (Pharmacia, Sweden), 0.2 μmol of primer, 20 ng of DNA, and 1 U of Taq polymerase]. The amplification products were separated by electrophoresis in 2% agarose and 6% PAG gels. Two hundred RAPD primers were used, 30 of them contained decamer and 170 were 18–23-mer sequences. Twenty ISS primers that were di- and trinucleotide repeats were also used.

Preparative quantity of a DNA fragment designated as a RAPD marker was isolated by electroelution from 2% agarose gel followed by reprecipitation with 96% ethanol from 3 M sodium acetate [7]. On the basis of the determined structure of the RAPD fragment, we constructed primers for the STS analysis with the use of Oligos program (W. Rychlik, United States).

For sequencing, the PAPD fragment was preliminarily cloned into the pGEM-T T-vector using a pGEM-T Easy Vector System kit (Promega, United States). The DNA fragment was ligated with vector pGEM-5Zf(+) digested with *Eco*RV in a polylinker followed by a TTP completion at the 3'-ends. The *E. coli* transformation was carried out as described in [7]. The clones containing the insert were selected on a medium containing IPTG and *Xga*l. The insert was sequenced in the Institute of Biotechnology of Helsinki University (http://www.biocenter.helsinki.fi/bi/dnal) using Dyenamic ET terminator kits with Thermo Sequenase™ DNA polymerase on a MegaBace™ 1000 DNA Sequencing System and Sequence Analyzer software (http://www.megabase.com/products/MegaBace/systeminfo.htm).

RESULTS AND DISCUSSION

Some differences in DNA amplification spectra of *Vrn-D1* genotypes in comparison with the amplification products of the genotypes bearing recessive alleles of *vnr* genes and dominant alleles *Vrn-A1* and *Vrn-B1* were revealed by means of the ISSR and RAPD analyses.

In the case of the ISSR analysis with the $(AGC)_6G$ primer, one of the reaction products (850 bp) was absent regardless of the gene pool of recurrent parents, which indicated the polymorphism of DNA monogenic lines with the dominant *Vrn-D1* locus (Fig. 1) [8]. The absence of this amplicone was also noticed in the spring wheat of the F_2 population, in particular, in the plants with early earing. The ISSR analysis of the DNA of wheat varieties of various ecological and geographical origins showed the absence of the 850-bp amplification fragment in the monogenic varieties under the dominant *Vrn-D1* control (Beacon, Santa Catalina, and Cocoraque F75).

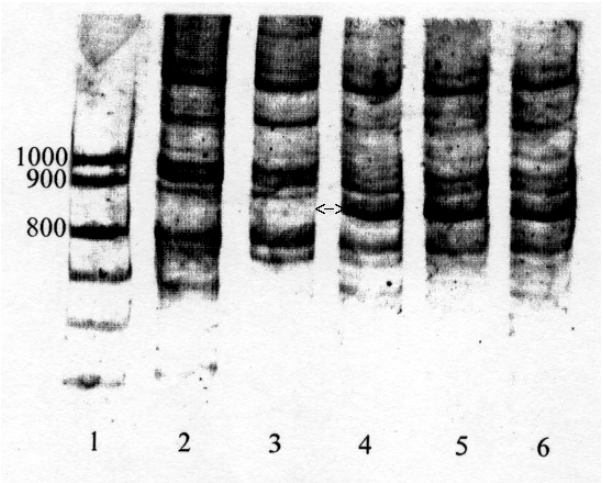

Fig. 1. Electrophoregram of the products of the PCR ISSR variant: *1*, molecular mass markers; *2* and *3*, monogenic *Vrn-D1*-dominant line of Mironovskaya 808 variety; *4*, monogenic *Vrn-B1*-dominant line of Mironovskaya 808 variety; *5*, monogenic *Vrn-A1*-dominant line of Mironovskaya 808 variety; and *6*, a recurrent parent of Mironovskaya 808 variety. The polymorphic fragments, *Vrn-D1* gene markers, are indicated by arrows.

The electrophoregrams of the amplification products obtained in an RAPD analysis with primer 116 (GGG CCA ACA CTG GAA CAC GAC ACG CCA CCG) demonstrate the presence of a 657-bp polymorphic amplicone in the monogenic lines with the dominant *Vnr-D1* locus; the lines were created in the gene pools of four winter soft wheat varieties. An RAPD analysis of the plants of F_2 population revealed 657-bp polymorphic amplicone in the spring forms and its absence in the winter forms. The results of the hybridological analysis of the F_2 population according to the type of development (summer or winter plants) and the data of the RAPD analysis on the presence/absence of the 657-bp amplicone correspond to the theoretically expected splitting at a 3 : 1 ratio in accordance with the χ^2 criterion. These results have been analyzed using the MARMARKER program in order to find the linkage of the RAPD marker with the *Vnr-D1* gene (for the detailed results, see [9]). An RAPD analysis of the DNA of spring wheat varieties of various ecological and geographical origins demonstrated that the 657-bp polymorphic amplicone is only present in the varieties bearing the *Vrn-D1* gene. The amplification reaction with primer 116 carried out with the material of null5/tetra5-substituted lines of the Chinese Spring variety indicated considerable changes in the PCR spectra of products of the lines with the substituted 5D chromosomes.

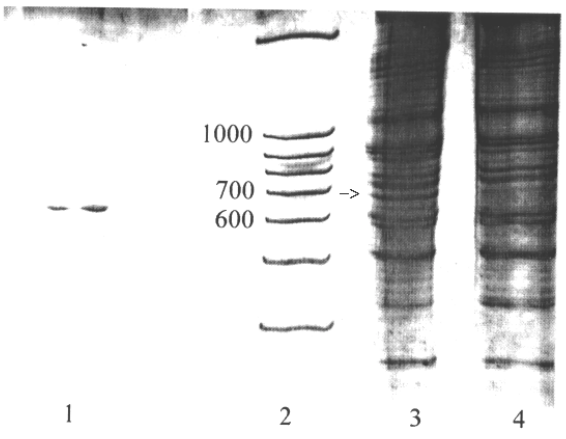

Fig. 2. Electrophoregram of the DNA amplification products with primer 116 in the presence of the DNA fragment eluted from 2% agarose gel: *1*, 657-bp RAPD fragment eluted from 2% gel; *2*, molecular mass markers; *3*, monogenic *Vrn-D1*-dominant line of Mironovskaya 808 variety; and *4*, a recurrent parent (Mironovskaya 808 variety). The polymorphic fragments, *Vrn-D1* gene markers, are indicated by an arrow.

The design of the STS marker to the *Vrn-D1* gene. The results of sequencing of the 657-bp polymorphic amplicone eluted from the agarose gel (Fig. 2) are shown in Fig. 3.

The use of the Oligos program enabled the choice of primer pairs directed to the terminal sites of the sequenced DNA fragment.

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GGG CCA ACA CTG GAA CAC GAC ACG CCA CCG CGT CTT CGT
CGA GGT GCC AGC ACA ATA CGA CAT CCA CAC CGA CAA CGA
TGT TCT AGT CAA CTC AGT CAT CGC CAC AGC ATT CTA CCC
CAA GAT CCT GAC CCG CGA AGG CAA GGG CTG GCG AAA CAT
CTC AAA CAA CCA AAC CGT CAG CCT AGG GCC AAC CTC CGT
CAA CAA AGG CTC GCG AAC AGC CGA CTT CCT ATC CTA CTA
CAA CAT CAT GCA ATC CAG CAA CAA GTT CTA CAA CGC GCA
CTC GAC CTC AAT CGC CTA CCC ACT TCC CAT GGT CCT CAT
GGT CGC TGC CGA TAT GGA CTT CAA GCT TCA TGC AGG CGT
CAT CAG TCT ACC CGG CAA CAT CAT CCG CTT CGG AGT TCG
TGA ATG GCG GGC TGC TGT CGC GCT GAA GGT ACT GAG ACG
CCG TAT CAA GGA GAT TCT GGC GAA TAG CTG GAA GAA CCC
TGC GCG TCA GCT GAG CGA TCG CGA GAA GGA GTG GCT TGG
CTT GTT CTA TAG GAT GTG GAG GAG AGG TTT GAG AAG GAC
GAG AGG ATT AGG AGA GGG CTG TGG AAG GCG AAG TGA GGA
TTG GGT TTG GTT GGA ATA TGT TAG ATG GAT CAC TTG TAG
ATG TGG ATT GTG TTG GTG TTC CAG TGT TGG CCC
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Fig. 3. Nucleotide sequence of the RAPD marker to the *Vrn-D1* locus

STS analysis of wheat genotypes. The STS analysis of the *Vnr-D1*-isogenic lines and their recurrent parents of winter soft wheat (varieties Mironovskaya 808, Odesskaya 16, Skorospelka 36, and Triple Dirk) enabled to reveal a polymorphism among these genotype groups. The spectra of the STS PCR products point out to the presence of a 780-bp amplicone in monogenic lines with dominant *Vrn-D1* gene regardless of the gene pool of recurrent parents. The presence of a 820-bp amplification product is inherent in the genotypes bearing the recessive alleles of *vnr* genes; this is absent in the monogenic genotypes with the dominant *Vrn-D1* gene. The presence of the 780-bp amplicone in the H5A/T5B and H5B/T5D lines and its absence in the H5D/T5B and H5D/T5A lines was observed when DNA of null5/tetra5-substituted lines of Chinese Spring variety that bears dominant alleles of the *Vrn-D1* gene was subjected to the STS analysis.

The availability of the corresponding genetic material facilitates the construction of DNA markers to individual genes. We selected DNA markers using the *Vrn*-isogenic lines. PCR analysis helps reveal the DNA sites linked to the regions that differentiate these lines. The use of such material decreases the probability of errors when interpreting the PCR results. The DNA polymorphism in this or that isogenic line (i.e., the presence or absence of one of the reaction products) could most likely be regarded as a potential DNA marker to the corresponding locus. The presence of a polymorphic amplicone may be due to distortions in nucleotide sequences of the primer site region that is not directly related to the locus under study. In this connection, some sets of isogenic lines designed in several gene pools of winter soft wheat are necessary. The presence of polymorphic amplicones in several isogenic lines with similar *Vrn* genotypes gives telling arguments to regard this polymorphic amplicone as a molecular marker.

The benefits of multilocus PCR analysis (RAPD or ISSR) were used to construct DNA markers to the *Vrn-D1* gene. The amplification reaction was carried out with ISSR primers that are simple repeated sequences with a 3'-terminal anchor composed of one or two randomly chosen nucleotides. The amplification with primer $(AGC)_6G$ demonstrated a possibility of marking the lines that bear the *Vrn-D1* genotype. The polymorphism proved itself as the lack of an 850-bp reaction product in the monogenic line with the dominant *Vrn-D1* locus (Fig. 1). The absence of the fragment could result from a mutation in the primer site, which also allowed us to regard the lack of the fragment as a molecular marker to a certain gene locus. The ISSR analysis of spring soft wheat DNA indicated the lack of an 850-bp amplicone only in monogenic *Vrn-D1*-dominant varieties Beacon, Santa Catalina, and Cocaraque F75. The electrophoregrams of monogenic varieties bearing the dominant allele of the *Vrn-A1* or *Vrn-B1* gene (Bledsol, ANK 18A) as well as digenic Erythrospermum 841, Sonalica (with dominant *Vrn-A1* and *Vrn-D1* genes) or Kentana (with dominant *Vrn-B1* and *Vrn-D1* genes) did not show the absence of the allele marker. The absence of the 850-bp amplicone was also shown in spring plants of the F_2 population (Mironovskaya *Vrn-D1* x Mironovskaya 808 recessive in *vrn-D1* gene) characterized by early earing. Usually, the genetic effects of the *Vnr* loci are obviously expressed [10], and one can suggest that early earing is inherent in dominant homozygotes; in our case, homozygotes with the dominant *Vrn-D1* gene. For spring plants with late earing (the *Vnr-D1* locus heterozygotes) and winter forms (with the recessive *vrn-D1* gene), the absence of 850-bp amplicone was not observed. The ISSR analysis with primer $(AGC)_6G$ allowed the identification of *Vrn-D1* in the wheat lines under the monogenic dominant *Vrn-D1* control.

The polymorphism identifying this or that isogenic line was observed when the RAPD method was used, but the 657-bp polymorphic amplicone, which can be detected in the *Vnr-D1*-isogenic lines designed in the gene pools of four winter wheat varieties, was observed only with the use of the random primer 116 (GGG CCA ACA CTG GAA CAC GAC ACG CCA CCG). The possibility of using a polymorphic RAPD fragment as a marker to the *Vrn-D1* gene was confirmed when the DNA from 52 spring wheat varieties of the F_2 population of various ecological and geographical origin and the null/tetrasomal lines of the Chinese Spring variety were analyzed. The DNA analysis of spring wheat varieties showed the presence of a 657-bp amplicone only in the genotypes bearing *Vrn-D1*. Both lines under the monogenic *Vrn-D1* control and digenic lines under the *Vrn-A1*/*Vrn-D1* or *Vrn-B1*/*Vrn-D1* control can be marked. The dominant alleles *Vrn-A1* and *Vrn-B1* do not affect the *Vrn-D1* gene detection. The RAPD and ISSR markers are limitedly useful, because they do not allow the detection of heterozygote forms. Since our RAPD marker was closely linked with the *Vrn-D1* gene, the nucleotide sequence of this amplicone was used for the design of STS marker to the gene under study. The STS analysis showed that the 820-bp and 780-bp fragments are amplified in the lines that bear the recessive *vrn* alleles (winter wheat varieties) and monogenic lines with dominant *Vrn-D1*, respectively. Both STS fragments that characterize the *Vrn* genotypes of the parent forms were the reaction products when DNA of F_1 population (*Vrn-D1* heterozygotes) was analyzed by STS method (Fig. 4). Hence,

the 780-bp fragment can serve as a STS marker for the homozygote plants with the dominant *Vrn-D1*, whereas the heterozygosis is detected by the presence of 820- and 780-bp amplicones. The localization of the STS marker on 5D chromosome was confirmed by the analysis of null/tetrasomal lines of the Chinese Spring variety. The spectra of the STS PCR products imply the presence of a 780-bp amplicone for the plants with the normal and doubled 5D chromosome number (H5A/T5D and H5B/T5A), since the Chinese Spring variety is under the monogenic control of *Vrn-D1*. The spectra of DNA amplification products for the lines with substituted 5D chromosomes (H5D/T5A and H5D/T5B) show the absence of the 780-bp amplicone, which is the marker of the *Vrn-D1* gene (Fig. 5). The lack of this fragment implies the lack of 5D chromosome.

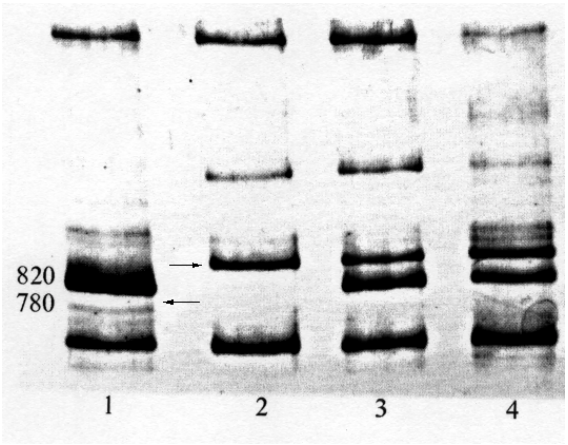

Fig. 4. Electrophoregram of the products of the STS PCR of parent wheat forms and hybrids F_1: *1*, a *Vrn-D1*-dominant homozygote; *2*, a *vrn-d1*-recessive homozygote; *3* and *4*, *Vrn-D1/vrn-d1* heterozygotes. The polymorphic fragments, *Vrn-D1* gene markers, are designated with arrows.

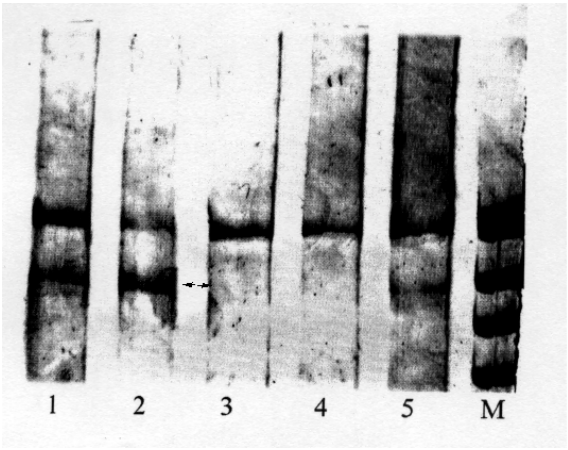

Fig. 5. Electrophoregram of the products of the STS PCR of null5/tetra5-substituted lines of Chinese Spring variety: *1*, H5A/T5B; *2*, H5B/T5D; *3*, H5D/T5B; *4*, H5D/T5A; and *5*, H5B/T5A. The polymorphic fragments, *Vrn-D1* gene markers, are designated with arrows.

To conclude, we developed a detection technology of wheat genotypes with the help of the *Vrn-D1* gene using PCR markers.

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