Supporting Online Materials (SOM)

Genetic and Molecular Characterization of the *VRN2* loci in Tetraploid Wheat

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Methods

Sequencing and annotation of the *VRN-B2* region

BAC clone 738D05 was selected from the BAC library of tetraploid wheat variety Langdon RSL#65 (Cenci et al., 2003) and was partially sequenced at low coverage and annotated. The *Triticeae* Repeat Sequence Database (TREP) was used to annotate the repetitive elements (http://wheat.pw.usda.gov/ITMI/Repeats/index.shtml). The non-repetitive sequence was further characterized by performing BLAST searches to GenBank nr and EST databases. Only the four contigs including genes (101,933-bp) were deposited in GenBank under accession numbers FJ173819 (*ZCCT-B1* gene, 17,652 bp), FJ173823 (*ZCCT-B2a* gene, 21,325 bp), FJ173824 (*ZCCT-B2b* gene, 9,722 bp), and FJ427399 (*SNF2P* gene, 53,234 bp).

Polymerase chain reaction (PCR)

Primers were designed using the PRIMER3 program (Rozen and Skaletsky, 2000). PCR reactions were carried out in a 20 µl reaction volume under the following conditions: initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and a final extension cycle of 72°C for 7 min.

Quantitative Reverse Transcription PCR analysis (q-RTPCR)

RNA was extracted from leaves of 3, 4 and 5-weeks old plants using Spectrum plant total RNA kit (Sigma-Aldrich, Saint Louis, MO). First strand cDNA was synthesized from 1 µg of total RNA with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA).

For q-RTPCR amplification, primer sets were designed from the sequences of *ZCCT1* and *ZCCT2* (Table SI, supporting online materials). These primers were specific for each gene but conserved for the A and B genome copies of each gene. Therefore, transcript levels for each gene are from both homoeologous copies. Quantitative PCR was performed on an ABI PRISM 7000 SDS (Applied Biosystems, Foster City, CA, USA) using SYBR® GREEN. PCR setup and reaction conditions were as reported before (Fu et

al., 2007). The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the endogenous wheat *ACTIN* control amplified with the primer sets specified in Table SI (supporting online materials).

Hybridization procedures

A tetraploid wheat BAC library (Cenci et al., 2003) and a BAC library from *Ae. tauschii* (Akhunov et al., 2005) were screened by hybridization using a *VRN2* probe. Positive BAC clones were confirmed by PCR and organized into two groups by hybridization of *Hind*III fingerprint Southern Blots. To establish the genome origin of the BAC clones, genome specific primers were designed from each contig and were then tested in nulli-tetrasomic lines missing chromosomes 5A (*VRN-A2*) and 4B (*VRN-B2*) (Sears, 1966).

For the RFLP germplasm screening, *Dra*I digested DNAs were transferred by Southern Blot to positively charged Nylon membranes and were hybridized with a probe including the second exon of the *ZCCT1* gene (*VRN2*). The germplasm accession included in these membranes has been described before (Dvorak et al., 2006). The origin of each restriction fragment was inferred by comparing the sizes of the hybridizing fragments with the predicted sizes of *Dra*I segments including the different *ZCCT* genes from the available genomic sequence (Fig. 2). Hybridizations procedures used for the screening of the BAC library and for the RFLP germplasm screening were as described before (Dubcovsky et al., 1994).

Molecular markers used in this study

***VRN-A^{m}2* marker**: A codominant CAPS marker was developed to detect the introgression of the recessive *vrn-A^{m}2* allele from *T. monococcum* DV92 into hexaploid wheat. Conserved primers VRN2/A2/F4 and R4 (Table SI) were designed to amplify orthologous 993 (*T. monococcum*) and 994-bp (A genome polyploid wheat) fragments from the *ZCCT-A2* intron. A polymorphic *Alu*I restriction site present in *T. monococcum* but not in the A genome of polyploid wheat was used to differentiate the two alleles in agarose gels (Fig. 3C). After *Alu*I digestion the A genome allele from polyploid wheat showed a 615-bp fragment whereas the *T. monococcum* allele showed two fragments of 541 and 74-bp (in addition to common fragments of 7, 13, 24, and 334/333- bp).

***VRN-B2* marker (*SNF-B2*)**: Since both copies of *ZCCT-B2* are deleted in *T. turgidum* ssp. *dicoccon* accession PI470739, the *ZCCT* genes cannot be used to develop a codominant marker, which is required for the selection of heterozygous *Vrn-B2/vrn-B2* plants. Therefore, a marker was developed for the linked gene *SNF-B2* which is located in the same BAC as *ZCCT-B1* and *ZCCT-B2* (738D05). Primers SNF2/B/F1and R1 (Table SI) were used to amplify a 944-bp product. Digestion of the amplified product with restriction enzyme *Hpy*CH4IV yielded two fragments of 589 and 355 bp in *T. turgidum* ssp. *dicoccon* accession PI470739 and an uncut 944-bp fragment in tetraploid varieties Langdon and Durelle alleles (Fig. 3I).

***VRN-A1* marker**: A CAPS marker was develop to test the presence of the *vrn-A^{m}1* allele from *T. monococcum* in the critical line BC_3F_2-521. Primers AP1_A_vs_DV92_F2 and R2 amplified an 815-bp fragment (3mM $MgCl_2$, 55°C annealing, 40 cycles). Digestion of this fragment with restriction enzyme *Pst*I yielded a 421-bp fragment in tetraploid varieties Langdon or Durelle and two fragments of 264 and 157-bp in *T. monococcum* DV92 (plus a common fragment of 394-bp in both alleles).

***PINA* marker**: A *PINA* marker developed before (Bonafede et al., 2007) was used to determine the presence of chromosome 5A^{m}S from *T. monococcum* in BC_3F_2-521. The *PINA* gene is deleted in tetraploid wheat.

Table SI. Primers and genetic markers.

Primer name	Target	SEQUENCE	PCR size bp
VRN2/B1/F3	*ZCCT-B1*	GCACCTCTGAATGAAAATGGA	797
VRN2/B1/R6		TGGTTACATATTATTTTTCCAGTATGA	
VRN2/B2/F2	*ZCCT-B2*	ATACATATGTCCGCGCCTTC	1106/1107
VRN2/B2/R5		TAACTCCTCCAACCGGTCAA	
VRN2/22F	*ZCCT1*	TCGTCATCACCATCATCAGG	135/
VRN2/22R		TGAAATGGTGTGGTCCATGT	156
VRN2/40F	*ZCCT-A1*	GTTCAGGTGCCATTTTACGG	657
VRN2/A1/R5		AACTGGTTAATTATTTTTCCAATATGA	
VRN2/40F	*ZCCT-B1*	GTTCAGGTGCCATTTTACGG	658
VRN2/B1/R6		TGGTTACATATTATTTTTCCAGTATGA	
AP1_A_vs_DV92_F2	*VRN-A1*	CAGCTGGAAAGCTCACTGAA	815
AP1_A_vs_DV92_R2		CCAAAGCGAACACAAGCATA	
Pina-A^{m}1F	*PINA*	GGTCAATCCAAGGCGACCTCA	306 [1]
Pina-A^{m}1R		GATTAACACAGGCATACTGAA	
SNF2/B/F1	*SNF2-B2*	AACGCTTTATGATGCCAAGG	942 [2]
SNF2/B/R1		TGTGGACAGAACTGGTTTGC	
VRN2/A2/F4	*ZCCT-A2*	AAAAAGTTAGCGCCATGTAACC	994 [3]
VRN2/A2/R4		CTAATAGTGCTGGTGAATGCAG	
Q-PCR_ZCCT1_F	*ZCCT1*	ATCACCTTCGCTGCTCTCTC	133
Q-PCR_ZCCT1_R		CCCACATCGTGCCATTTTAC	
Q-PCR_ZCCT2_F	*ZCCT2*	CCACCATCGTGCCATTCT	88
Q-PCR_ZCCT2_R		CCCACCATCATCTCTGTATCAA	
Actin F		ACCTTCAGTTGCCCAGCAAT	91
Actin R	*ACTIN*	CAGAGTCGAGCACAATACCAGTTG	

[1] Dominant marker. The 306-bp product is only amplified from the *T. monococcum* allele.

[2] Digested with *Hpy*CH4IV.

[3] Digested with *Alu*I.

References for supporting online materials

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