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ORIGINAL PAPER

Development and validation of a *Viviparous-1* STS marker for pre-harvest sprouting tolerance in Chinese wheats

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Abstract Pre-harvest sprouting (PHS) of wheat reduces the quality of wheat grain, and improving PHS tolerance is a priority in certain wheat growing regions where conditions favorable for PHS exist. Two new *Viviparous-1* allelic variants related to PHS tolerance were investigated on B genome of bread wheat, and designated as *Vp-1Bb* and *Vp-1Bc*, respectively. Sequence analysis showed that *Vp-1Bb* and *Vp-1Bc* had an insertion of 193-bp and a deletion of 83-bp fragment, respectively, located in the third intron region of the *Vp-1B* gene. The insertion and deletion affected the expression level of the *Vp1* at mature seed stage, more correctly spliced transcripts were observed from the genotypes with either insertion or deletion than that of the wild type. Based on these insertions and deletions, a co-dominant STS marker of *Vp-1B* gene was developed and designated as *Vp1B3*, which in most cases could amplify either 845 or 569-bp fragment from the tolerant cultivars, and 652-bp from the susceptible ones. This *Vp1B3* marker was mapped to chromosome 3BL using a set of Chinese Spring nulli-tetrasomic and ditelosomic lines. A total of 89 white-grained Chinese wheat cultivars and advanced lines, were used to validate the relationship between the polymorphic fragments of *Vp1B3* and PHS tolerance. Statistical analysis indicated that *Vp1B3* was strongly associated with PHS tolerance in this set of Chinese germplasm, suggesting that *Vp1B3* could be used as an efficient and reliable co-dominant marker in the evaluation of wheat germplasm for PHS tolerance and marker-assisted breeding for PHS tolerant cultivars.

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Introduction

Pre-harvest sprouting (PHS) is defined as the germination of grains in the ear before harvest (Groos et al. 2002). It is a widespread response to climatic conditions and occurs in areas as diverse as Canada, the USA, Europe, Australia, China and Japan (Derera 1990). PHS reduces the quality of wheat and the economic value of the grain. The flour of sprouted grain has a lower falling number because the active α-amylases degrade the starch, resulting in poor baking quality (Humphreys and Noll 2002). PHS tolerance is influenced by many environmental factors and controlled by several dormancy-related genes and QTLs (Bailey et al. 1999; Flintham 2000; Mares et al. 2005; Tan et al. 2006).

Dormant cultivars are not able to germinate and grow under conditions favorable for non-dormant cultivars; therefore seed dormancy is a major factor that controls the PHS under wet weather condition (Li et al. 2004). The identification and use of molecular markers associated with PHS should accelerate the production of PHS tolerant cultivars, as it is difficult to establish reliable screening conditions for selection during breeding. Using a doubled haploid population of bread wheat, a major quantitative trait locus (QTL) for dormancy was located on the long arm of chromosome 4A by simple sequence repeat (SSR)-based genetic mapping. This QTL explained 43.3% of phenotypic variation for seed dormancy under greenhouse condition, but its effect was not significant under the field condition (Atsushi and Yoichi 2002). A STMS (sequence-tagged microsatellite site) marker *WMC104* and a STS (sequence-tagged site) marker *MST101*, located on the chromosomes 6B and 7D, respectively, were identified as showing strong association with tolerance to PHS (Roy et al. 1999). Another two QTLs related to PHS were mapped to chromosomes 4AL and 3AL, using a set of recombinant inbred lines from the ITMI mapping population. However, these two markers need to be validated under a range of environmental conditions (Lohwasser et al. 2005). In addition, two significant QTLs for grain dormancy were located on chromosome 4AL and 5BL, respectively (Mares et al. 2005; Tan et al. 2006). While 4AL QTL has been reported in wheat cultivars of diverse origin and was found to be strongly influenced by the environment (Kato et al. 2001; Mares et al. 2005; Tan et al. 2006), the 5BL QTL associated with about 10% of the total phenotypic variations was found to be remarkably consistent (Tan et al. 2006).

The viviparous (*Vp-1*) gene is an important regulator of late embryogenesis in maize and a regulator of late embryo development in bread wheat (McCarty et al. 1991). The phenotypes of the *Vp-1* mutants of maize showed that *Vp-1* performs two distinct functions: one is to promote embryo maturation, and the other is to advance embryo dormancy and simultaneously to repress germination (McCarty et al. 1991). *Vp-1* has been cloned and sequenced from several plant species (McKibbin et al. 2002; Jones et al. 1997; Giraudat et al. 1992). Three orthologues *Vp-1* genes are present in bread wheat, which are located on the long arms of chromosomes 3A, 3B and 3D, respectively (Bailey et al. 1999). The structure and expression of the three *Vp-1* homologues in bread wheat have been determined, showing that each has the potential to encode a full-length functional protein. However, incorrect splicing of pre-mRNA led to a diverse RNA population that in most cases encoded aberrant translation products (McKibbin et al. 2002). The transcript structures in ancestral and closely related species were also analyzed and it was suggested that missplicing of *Vp-1* genes occurred before the evolution and domestication of bread wheat (McKibbin et al. 2002). In addition, the expression level of *Vp-1* was determined in mature embryos of dormant and no-dormant cultivars, indicating a positive correlation between seed dormancy and embryo sensitivity to ABA (Nakamura and Toyama 2001). In *Avena fatua*, expression of *AfVp1* is controlled by the interaction between the environment and genotype, with a close correlation between *AfVp1* mRNA levels and seed dormancy (Jones et al. 1997). Transgenic wheat seeds expressing the *AfVp1* cDNA showed increased dormancy and tolerance to PHS (McKibbin et al. 2002). However, no significant association with seed dormancy on chromosome group 3 was reported in populations of crosses between dormant AUS 1408 and non-dormant parents (Mares et al. 2005).

Breeding for PHS tolerant cultivars is important in the Northern and Northeastern as well as in the Yangtze River valley regions in China. The objectives of this study were to explore new allelic variability of *Vp-1* among Chinese landraces and cultivars with different level of PHS tolerance and to develop efficient markers for marker-assisted breeding. Furthermore, the identification of these new *Vp-1* resources could also contribute to our understanding of the mechanisms underlying PHS tolerance in bread wheat.

Materials and methods

Plant materials

Four well-known white-grained genotypes conferring different levels of PHS tolerance were selected and used for PCR amplification of *Vp-1A*, *Vp-1B* and *Vp-1D*. These were Zhongyou 9507 (PHS susceptible cultivar), Yongchuanbaimai and Wanxianbaimai (typical PHS tolerant Chinese landraces), and Xinong 979 (PHS tolerant cultivar).

Eighty-four white-grained wheat cultivars, advanced lines, and landraces were used to validate the developed STS marker *Vp1B3* in this study, as presented in Table 1. Among these selected 84 white-grained cultivars and lines, 17 white-grained cultivars, which have different levels of PHS tolerance, were employed at first to develop the new STS marker. All selected germplasm were grown in Zhengzhou (Henan) and Beijing in 2000–2001 and 2001–2002 under normal field management. Each plot consisted of 2 m rows spaced 0.3 m apart. The trial was kept free of weeds and diseases, with two applications of broad-range herbicides and fungicides. These genotypes, including released cultivars, historical wheats and landraces were selected from different parts of China, and could well represent the current diverse levels of PHS tolerance of Chinese wheats (Table 1).

Table 1 Polymorphism of the *Vp1B3* marker in the selected 89 white-grained cultivars with different level of PHS tolerance

No	Cultivar	GI × 100			PCR fragment size (bp)	No	Cultivar	GI × 100			PCR fragment size (-bp)
		2001	2002	Average				2001	2002	Average	
1	Anxuan 2	5	3	4.0	569	46	Fengkang 13[a]	97	100	98.5	652
2	Baikezao 2	9	1	5.0	569	47	Fengkang 4	79	100	89.5	652
3	Baituzi[a]	24	1	12.5	569	48	Hongsuibai	17	10	13.5	652
4	Baiyupi	7	8	7.5	569	49	Jimai 1	90	100	95.0	652
5	Bamai 5	46	52	49.0	569	50	Jimai 14	98	100	99.0	652
6	Bima 1	16	9	12.5	569	51	Jimai 21[a]	84	96	90.0	652
7	Chuan 81–11108	2	.	2.0	569	52	Jimai 9	97	99	98.0	652
8	Cuannong 94-DH343[a]	7	4	5.5	569	53	Jing 411	65	98	81.5	652
9	Fengchan 3	34	32	33.0	569	54	Jing 437	91	99	95.0	652
10	Huixianhong[a]	4	26	15.0	569	55	Jinghe 3	99	96	97.5	652
11	Jinghua 1	76	98	87.0	569	56	Jingshuang 10	90	97	93.5	652
12	Jingyang 302	54	22	38.0	569	57	Jinmai 10	68	100	84.0	652
13	Kaifeng 124	12	2	7.0	569	58	Jinmai 5[a]	96	99	97.5	652
14	Lumai 1	36	43	39.5	569	59	Keyi 26[a]	97	96	6.5	652
15	Lumai 21	11	6	8.5	569	60	Lanhuamai	10	7	8.5	652
16	Luoyangdakoumai	13	9	11.0	569	61	Linfen 7023	33	45	39.0	652
17	Mianyang 15	0	0	0.0	*569*	62	Lumai 12	40	27	33.5	652
18	Mianyang 8186-0-14	4	6	5.0	569	63	Lumai 7	42	26	34.0	652
19	Mianyang 93–352	7	6	6.5	569	64	PH 8516	56	28	42.0	652
20	Mianyang 94–315	2	13	7.5	569	65	Shandong 928802	51	42	46.5	652
21	Nanda 2419[a]	5	4	4.5	569	66	Shannongfu 63	38	33	35.5	652
22	Neixiang 173[a]	4	4	4.0	569	67	Shijiazhuang 407	63	24	43.5	652
23	Neixiang 19	6	6	6.0	569	68	Shijiazhuang 54[a]	77	100	88.5	652
24	Pingyuan 50	11	1	6.0	569	69	Xiaoyan 22	62	46	54.0	652
25	Qida195	13	8	10.5	569	70	Xinong 88	32	47	39.5	652
26	Shannong 6521	1	11	6.0	569	71	Xuyongbaimai[b]	–	–	<8.0	652
27	Shannong 9[a]	11	15	13.0	569	72	Yibinbaimai[b]	–	–	<8.0	652
28	Shanxi 54[a]	6	6	6.0	569	73	Yuandong 3	68	99	83.5	652
29	Shuwan 761[a]	0	1	0.5	569	74	Yuanfeng 1	55	45	50.0	652
30	SW 95–16117[a]	3	2	2.5	569	75	Yumai 15	70	31	50.5	652
31	Xiaoyan 168	5	11	8.0	569	76	Yumai 21	62	42	52.0	652
32	Xiaoyan 5	2	7	4.5	569	77	Yumai 62	52	42	47.0	652
33	Xichang 5762	5	6	5.5	569	78	Zhengzhou 3	46	53	49.5	652
34	Xinong 6028	6	10	8.0	569	79	Zhengzhou 6	44	28	36.0	652
35	Xinyang 1	10	8	9.0	569	80	Zhoumai 13	94	63	78.5	652
36	Yumai 34	31	37	34.0	569	81	Biansuimai	50	45	47.5	845
37	Yumai 50	42	58	50.0	569	82	Chuanyu 21729[a]	10	5	7.5	845
38	Yumai 8	33	29	31.0	569	83	Fulingbaimai[b]	–	–	<8.0	845
39	Zaoyangmai	1	1	1.0	569	84	Jinan 4	41	47	44.0	845
40	Zhongmai 2	89	96	92.5	569	85	Liangzhongbaimai	–	–	<8.0	845
41	935031	80	61	70.5	652	86	Lumai 2	38	38	38.0	845
42	951741	49	43	46.0	652	87	Rongchangbaimai[b]	–	–	<8.0	845
43	CA9722[a]	81	97	89.0	652	88	Shannong 3[a]	40	24	32.0	845
44	Changle 5	63	41	52.0	652	89	Taishan 5	37	63	50.0	845
45	Dexuan 1	44	25	34.5	652						

The GI value of 84 cultivars presented in this table was evaluated by Dr. Shihe Xiao in 2001 and 2002 (Xiao et al. 2004)

[a] These 17 genotypes were used for the development of the STS marker *Vp1B3*

[b] These genotypes were planted in a different experiment, they showed consistent PHS tolerance with GI <8% over years and locations (Xiao et al 1986)

'–' Data not determined

No PHS or damages by rainfall was observed in the field in Zhengzhou or Beijing in years 2000–2001 and 2001–2002. Each cultivar was harvested separately when it reached the dough-yellow ripening stage. Germination index (GI) was calculated according to Walker-Simmons (1988) (See "Materials and methods"). Among this set of germplasm, 34 genotypes had germination index of less than 15.0%, 30 between 31.0 and 54.0%, whereas the other 20 more than 70.0%. Detailed information on germination index (GI) testing was published previously (Xiao et al. 2004).

In addition, another five well-known Chinese PHS tolerant landraces tested over years and locations, including Rongchangbaimai, Yibinbaimai, Liangzhongbaimai, Xuyongbaimai and Fulingbaimai with a germination index less than 8%, were also included to validate the STS marker. These genotypes have shown consistent PHS tolerance over years and locations (Xiao et al. 1986).

A set of Chinese Spring nullisomic–tetrasomic lines and ditelosomic lines 3BL and 3BS, provided by Prof. McIntosh from the University of Sydney, were used to verify the chromosomal location of the STS marker *Vp1B3*.

Methods

PHS tolerance assay

PHS tolerance was assessed based on germination index values. Ears were harvested at dough-yellow ripening stage, hand-threshed and sterilized with $HgCl_2$, and then placed crease down in plastic petri dishes on moist filter paper at room temperature. Germinated seeds were counted daily and removed. Germination index was calculated according to GI value (Walker-Simmons 1988):

$$\mathrm{GI} = \frac{7 \times n_1 + 6 \times n_2 + 5 \times n_3 + 4 \times n_3 + \ldots + 1 \times n_7}{7 \times \text{total number of grains}}$$

where n_1, n_2,...,n_7 are the number of seeds germinated on the first, second, and the subsequent days until the seventh day, respectively. Three replications for each sample were performed, with 100 seeds per testing. The GI values were computed by taking the average of data obtained over 2 years in two locations.

Primer design

Gene-specific primers were developed based on the DNA sequences of the three homologues *Vp-1A*, *Vp-1B* and *Vp-1D* that are available in GenBank (http://www.ncbi.nlm.nig.gov) under the accession numbers AJ400712, AJ400713 and AJ400714, respectively. Six pairs of primers, Vp-1AF_1/R_1, Vp-1AF_2/R_2, Vp-1BF_1/R_1, Vp-1BF_2/R_2, Vp-1DF_1/R_1, Vp-1DF_2/R_2 were designed to amplify fragments of the B_3 domains of the *Vp-1A*, *Vp-1B* and *Vp-1D* genes, respectively, in two cultivars (Zhongyou 9507 and Xinong 979) and two landraces (Wanxianbaimai and Yongchuanbaimai) with different level of PHS tolerance (Table 2). The primer RTVp-1BF/R was designed to perform RT-PCR analysis. Wheat *ACTIN* gene was used in this study as an internal control and included in each reaction in order to normalize the expression level of *Vp-1* genes, and the expected PCR product was 410 bp in length (Table 2).

Based on the amplified fragment of *Vp-1B*, an STS marker *Vp1B3* (Vp-1B_3F/R) that could amplify the polymorphic region was designed, validated and tested in Chinese wheats. Primers were also designed to amplify the complete sequence of *Vp-1B* (Fig. 1). The sequences of these primer sets are as shown in Table 2.

DNA extraction and PCR amplification

Genomic DNA was isolated from kernels using the method described by Gale et al (2001). For each genotype, three DNA samples, two from individual seeds and one from a composite sample from three seeds, were amplified in order to verify the purity of the sample. PCR reactions were performed in an MJ Research PTC-200 thermal cycler in a total volume of 50 µl including 5 µl 10× PCR buffer, 125 µM of each dNTP, 8 pmol of each primer, 2.0 U of *Taq* polymerase (TaKaRa Co. Ltd) and 100 ng of template DNA. The conditions for PCR amplification were 94°C for 5 min, followed by 36 cycles of 94°C for 1 min, 53–66°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 10 min. Amplified PCR fragments were separated on a 2.0% agarose gel, stained with ethidium bromide, and visualized using UV light.

RNA isolation from embryos and semi-quantitative RT-PCR analysis

Total RNA was extracted from the 35 DAP embryos as described by Chang et al. (1993). RNA concentration and quality were determined spectrophotometrically at 260 nm and by the A260/A280 ratio, respectively. RNA integrity was assessed by comparing the relative intensities of the 28 S and 18 S rRNA bands by electrophoresis in an agarose 1.2% (w/v) gel containing 2.2 M formaldehyde. cDNA was synthesized from 5 µg total RNA using M-MLV reverse transcriptase (TaKaRa) with random hexamer primer Oligo $d(T)_{18}$ according to the manufacture's instructions.

Semi-quantitative RT-PCR reactions were performed in an MJ Research PTC-200 thermal cycler in a total volume

Table 2 The primer sets used in this study

Primer set	Upstream	Downstream	Annealing (°C)	Fragment size (-bp)
Vp-1AF$_1$/R$_1$	5′-ATCCAAACCGGCGCTTCCCTCAAGA-3′	5′-CAAAATCGATCGATGGGAGTACTA G-3′	56	1,108
Vp-1AF$_2$/R$_2$	5′-AGGACATCGGCACATCTCA-3′	5′-CTGGTCAGTTTGCAACATGCAAC-3′	53	912
Vp-1BF$_1$/R$_1$	5′-ATCCAAACCGGCGCTTCCCTCAAGA-3′	5′-CTTACCGGTACCGCATGCTCCAG-3′	60	1,031
Vp-1BF$_2$/R$_2$	5′-AGGACATCGGCACATCTCA-3′	5′-CAAAATGGCAGCAACTGATCAGTTC-3′	55	960, 1,153, 877
Vp-1DF$_1$/R$_1$	5′-ATCCAAACCGGCGCTTCCCT CAA GA-3′	5′-GAACGTGCGTGTCCCACACAC-3′	60	1,214
Vp-1DF$_2$/R$_2$	5′-AGGACATCGGCACATCTC A-3′	5′-CCGCCTTATATTTTGATACGC-3′	60	1,025
Vp-1B$_3$F/R (*Vp1B3*)	5′-TGCTCCTTTCCCAATTGG-3′	5′-ACCCTCCTGCAGCTCATT G-3′	61	652, 845, 569
RTVp-1BF/R	5′-ATC CAA ACC GGC GGC TTC CCT CAA GA-3′	5′-CTT GTG CTT GGC TAG ATC CTG TTG A-3′	60	672
ACTIN up/down	5′-GTTTCCTGGAATTGCTGATCGCAT-3′	5′-CATTATTTCATACAGCAGGCAAGC-3′	62	410
Vp-1BB$_3$F/R	5′-ATGGACGCCTCCGCCGGCTC-3′	5′-CTGCTGCTGCAGGCACGACAA-3′	65	1,227
Vp-1BB$_4$F/R	5′-CAATGAGCTGCAGGAGGGTGA-3′	5′-ATCATCCCTAACTAGGGCTACG-3′	66	911

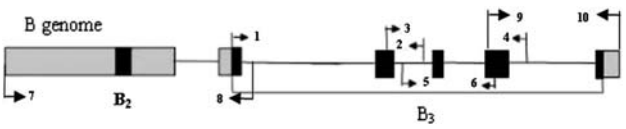

Fig. 1 Schematic diagram for the locations of primer sets used for *Vp-1B* gene amplification. Exons (*boxed*) and introns (*lines*) are shown, as are the position of the B2 and B3 domains within the coding regions (*black boxes*). 1/2- Vp-1BF$_1$/R$_1$, 3/4- Vp-1BF$_2$/R$_2$, 5/6- Vp-1B$_3$F/R, 7/8- Vp-1BB$_3$F/R, 9/10- Vp-1BB$_4$F/R

of 25 μl, using the protocol described by the instruction manual of the GC PCR kit (Clontech), including 1 μl of above cDNA template. The reaction conditions were 94°C for 5 min, followed by 36 cycles of 94°C for 1 min, 60–68°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 10 min. The RT-PCR products were separated on a 2.0% agarose gel. Values were normalized with the amplification rate of the *ACTIN* gene as a constitutively expressed internal control. Three replicates were performed for each sample.

Sequencing of PCR products

The PCR products were sequenced from both strands by Shanghai Songon Biological Technology Co. Ltd (http://www.sangon.com). Sequence analysis and characterization were performed using software DNAMAN (http://www.lynon.com).

Statistical analysis

Analysis of variance was conducted by PROC MIXED in the Statistical Analysis System (SAS Institute, 1997) with genotype cluster indicated by three types of fragments, which were amplified with STS marker *Vp1B3*, as a categorical variable to derive mean GI value for each cluster and to test significant levels. The genotypes cluster was treated as fixed effects, while genotypes nested in cluster and years as random. Pearson's linear correlation coefficients for GI between years were obtained by SAS PROC CORR.

Results

Amplification and sequence analysis of the three *Vp-1* homologues in four genotypes differing in PHS tolerance

After PCR amplification and sequencing analyses, no polymorphic fragments were detected within the *Vp-1A* and *Vp-1D* genes with the primer sets Vp-1AF$_1$/R$_1$,

Vp-1AF$_2$/R$_2$ and Vp-1DF$_1$/R$_1$, Vp-1DF$_2$/R$_2$, respectively, except that *Vp-1B* gene showed polymorphism in the four tested cultivars and landraces differing in PHS tolerance with the primer set Vp-1BF$_2$/R$_2$. The two landraces Wangxianbaimai and Yongchuanbaimai with higher levels of PHS tolerance, had a 193 bp insertion in the third intron region of the *Vp-1Ba*, while the newly released PHS tolerant cultivar Xinong 979 had an 83 bp deletion in the same area. Interestingly, these mutations were not present in Zhongyou 9507 that was susceptible to PHS (Fig. 2). The full length of the two *Vp-1B* alleles present in the PHS tolerant lines were further isolated with the primer sets Vp-1BB$_3$F/R and Vp-1BB$_4$F/R, and designated as *VP-1Bb* and *VP-1Bc*, respectively. Sequence analysis showed that these two new *Vp-1B* alleles had 95.43 and 97.89% similarity with the sequence of *Vp-1Ba* (AJ400713) at the nucleotide level. Their sequences were deposited in the GenBank under accession numbers of DQ517493 and DQ517494, respectively.

Expression characterization of the above three *Vp-1B* alleles at mature seed stage

The expression patterns of *Vp-1B* alleles at mature seed stage were investigated by semi-quantitative RT-PCR in

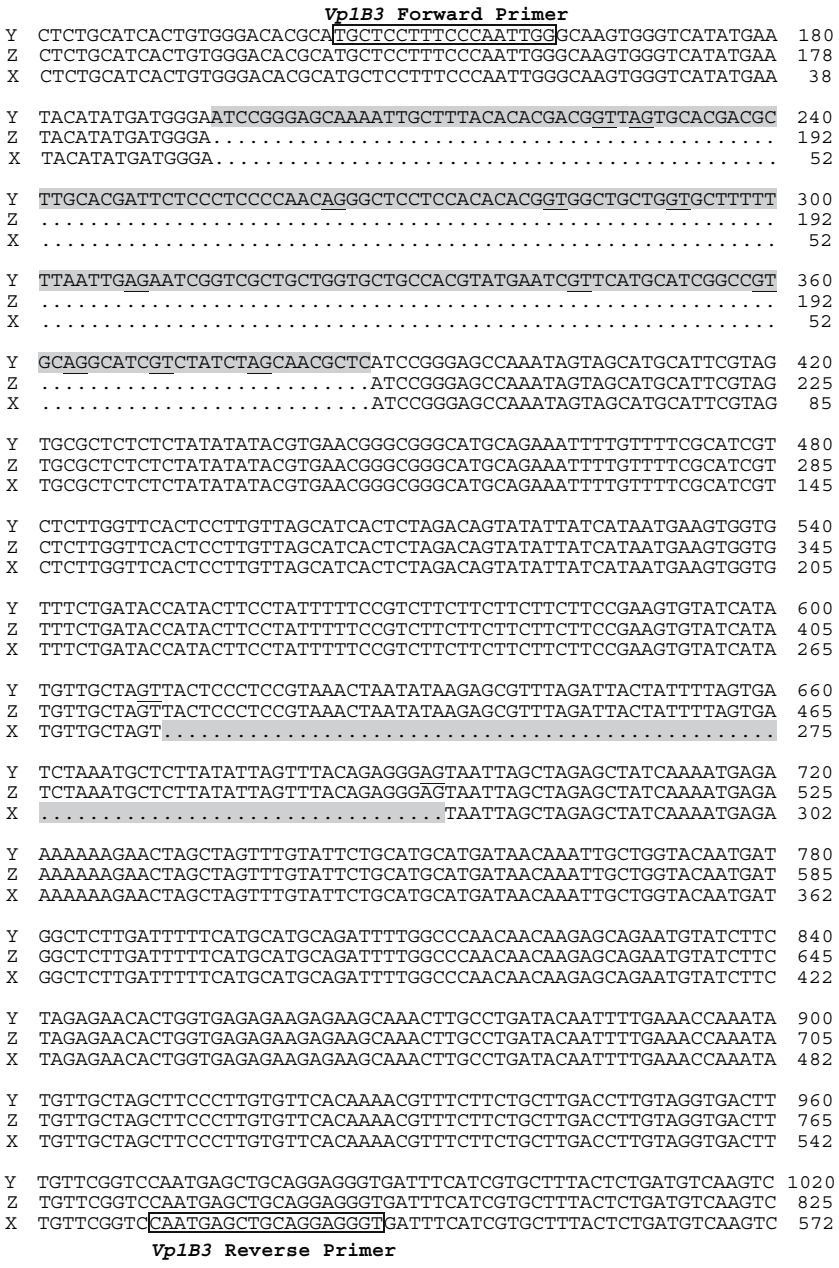

Fig. 2 Alignment on the partial *Vp-1B* genomic sequences from PHS tolerant landrace Yongchuanbaimai (Y) and cultivar Xinong 979 (X), and PHS susceptible cultivar, Zhongyou 9507 (Z). Sequence alignment indicated that, compared with Z, Y had a 193 bp insertion, while X had a 83 bp deletion. The *boxed sequence* showed the forward primer and reverse primer of *Vp1B3* marker, respectively. Sequences *underlined* indicated the postulated exon–intron boundary

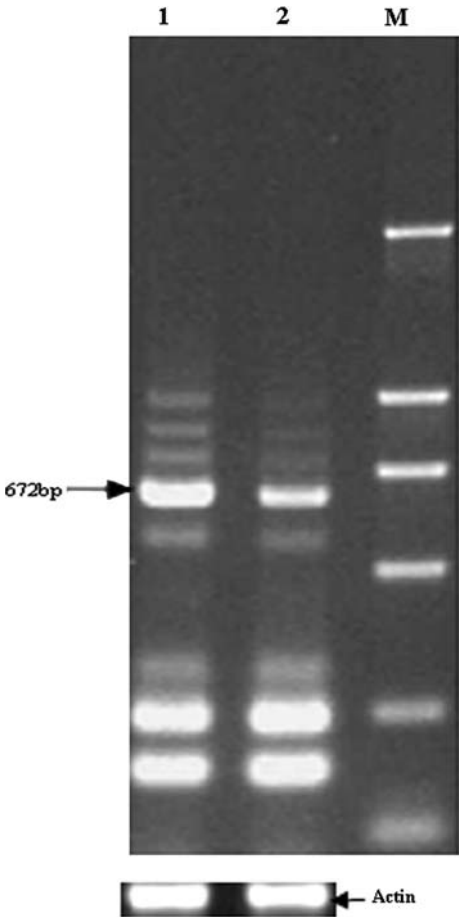

Fig. 3 The mis-spliced phenomena present both in PHS tolerance landrace and susceptible cultivars. **1** Yongchuanbaimai (*Vp-1Bb*); **2** Zhongyou9507 (*Vp-1Ba*); **M** D-2000 ladder. The *arrow* indicates the correctly spliced transcript, which has the potential to encode the full length Vp-1 protein

order to further understand their characterizations. As indicated in Fig. 3, in 35 DAP embryos, a set of transcripts were detected, among them, only the 672-bp fragment had the capacity to encode the whole length VP1 proteins, while the others did not. Although mis-spliced transcripts were present, majority of them were correctly spliced. For the correctly spliced transcript, *Vp-1Bb* had higher abundance than that of *Vp-1Ba*, showing close consistence with seed dormancy.

Development of the *Vp-1B* STS marker for PHS tolerance

Based on the sequence analysis, a *Vp-1* STS marker, designated as *Vp1B3* (Vp-1B$_3$F/R), was developed and used for PCR amplification of 17 Chinese cultivars and lines with different levels of PHS tolerance (Table 1). Three clearly different fragments were amplified, with 845-bp

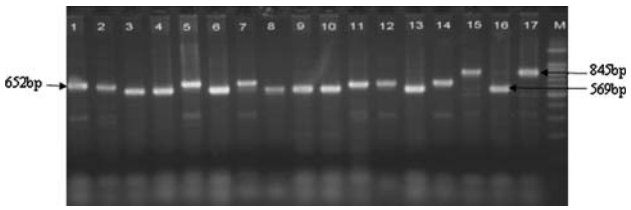

Fig. 4 PCR fragments amplified with *Vp1B3* in 17 Chinese wheat cultivars with different PHS tolerance. **1** CA 9722 (average germination index: 91.5%), **2** Fengkang 13 (98.5%), **3** Neixiang 173 (4.0%), **4** Shannong 9 (11%), **5** Jinmai 5 (97.5%), **6** Shuwan 761 (0.5%), **7** Shijiazhuang 54 (88.5%), **8** SW 95–16117 (2.5%), **9** Chuannong 94-DH343 (5.5%), **10** Baiyupi (7.5%), **11** Jimai 21 (90.0%), **12** Huixianhong (15.0%), **13** Shanxi 54 (6.0%), **14** Keyi 26 (96.5%), **15** Chuanyu 21729 (7.5%), **16** Nanda 2419 (4.5%), **17** Shannong 3 (32%), **M** 100 bp DNA ladder. Note: the number in the bracket indicated the GI values

fragments being amplified from Shannong 3 and Chuanyu 21729, 569-bp fragments from Nanda 2419, Shanxi 54, Baiyupi, Chuannong 94-DH343, SW 95–16117, Shuwan 761, Shannong 9 and Neixiang 173, and 652-bp fragments from Keyi 26, Huixianhong, Jimai 21, Shijiazhuang 54, Jinmai 5, Fengkang 13 and CA9722. Most interestingly, among these 17 cultivars and lines, all those exhibiting PHS tolerance had either 845 or 569-bp fragments, while 652-bp fragments were always present in PHS susceptible genotypes (Fig. 4). Analysis of the Chinese Spring nullitetrasomic lines and ditelosomic lines with 3BL and 3BS confirmed that this STS marker was located on chromosome 3BL (Fig. 5).

Validation of the *Vp1B3* marker with white-grained cultivars and lines with different levels of PHS tolerance

Eighty-nine white-grained cultivars and lines were used to validate this STS marker *Vp1B3*. The results of PCR amplification in some of the 89 cultivars were shown in

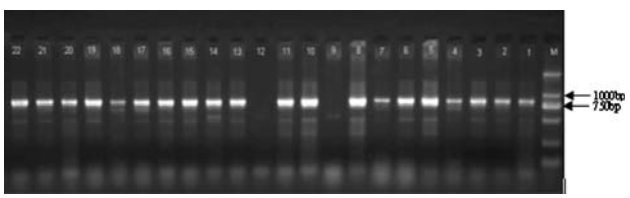

Fig. 5 PCR amplification of Chinese Spring nulli-tetrasomic lines and ditelosomic lines 3BL and 3BS using the STS marker *Vp1B3*. **M** D 2000 marker; **1** Chinese Spring nullisomic1A-tetrasomic 1D (N1A-T1D), **2** N1B-T1D, **3** N1D-T1B, **4** N2A-T2B (derived from M2A-T2B line), **5** N2B-T2A, **6** N2D-T2B, **7** N3A-T3D, **8** N3D-T3A, **9** N3B-T3D, **10** N4A-T4B, **11** ditelosomic line 3BL, **12** ditelosomic line 3BS, **13** N4D-T4B, **14** N5A-T5B, **15** N5B-T5A, **16** N5D-T5B, **17**‘ N6A-T6B, **18** N6B-T6D, **19** N6D-T6B, **20** N7A-T7D, **21** N7B-T7D, **22** N7D-T7A

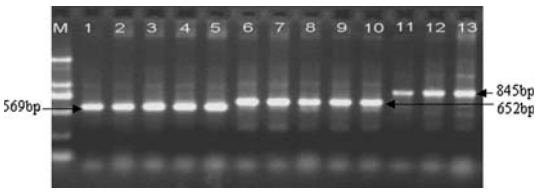

Fig. 6 Polymorphism of PCR fragment amplified with *Vp1B3* in 10 white-grained cultivars and landraces with PHS tolerance and 5 PHS susceptible cultivars. **M** D 2000 DNA marker, **1** Mianyang 15 (average germination index 0.0%), **2** Xiaoyan 5 (2.5%), **3** Neixiang 19 (6.0%), **4** Baituzi (12.5%), **5** Neixiang 173 (4.0%), **6** Jingshuang 10 (96.5%), **7** Zhoumai 13 (78.5%), **8** Jing 411 (81.5%), **9** Shijiazhuang 54 (88.5%), **10** Fengkang 13 (98.5%), **11** Yibinbaimai (<8%), **12** Langzhongbaimai (<8%), **13** Fulingbaimai (<8%). Note: the number in the bracket indicated the average GI values

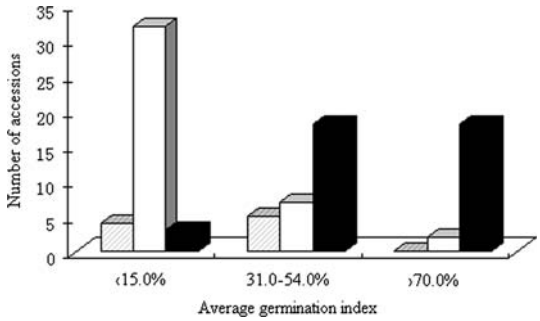

Fig. 7 Association between PHS tolerance (germination index) and the size of PCR fragments amplified with *Vp1B3* in 89 white-grained bread wheat cultivars and landraces. *White columns* indicate the number of accessions with 569 bp, *black columns* indicate the number of accessions with 652 bp, and *grey columns* indicate the number of accessions with 845 bp

Fig. 6, and the detailed results for 89 genotypes were presented in Table 1. The occurrence of the three PCR fragments amplified with *Vp1B3* in 89 white-grained cultivars was indicated in Table 3 and Fig. 7. Among the 89 genotypes, the 845-bp fragment was amplified in 9 genotypes including 4 PHS tolerance cultivars and 5 cultivars with intermediate PHS tolerance, and the 569-bp fragment was amplified in 39 genotypes including 30 PHS tolerance cultivars, 7 intermediate cultivars and 2 susceptible cultivars, and the 652-bp fragment was amplified in 39 genotypes including 3 PHS tolerance cultivars, 18 intermediate cultivars and 18 susceptible ones.

The germination index values (GI) of the 84 genotypes showed consistence over the 2 years ($R = 0.898$, $P < 0.0001$), with mean values and standard deviations were 39.9 ± 32 and 39.4 ± 36 in the year of 2001 and 2002, respectively. Analysis of variance indicated that there were significant differences among clusters for GI. The genotype cluster with 652-bp fragment showed more susceptible to PHS with average GI × 100 value of 65.5 than that of the other two clusters with either 569 or 845 bp, which showed tolerance to PHS with average GI × 100 value of 16.9 and 28.6, respectively. Therefore the genotypes with either 569 or 845 bp were more tolerance to PHS than the ones with 652 bp. It was concluded that the 845 and 569-bp fragments were always associated with PHS tolerance while the 652-bp fragment is more related to PHS susceptibility.

Discussion

It was proposed that *Vp-1A* had a very small effect of about 4% at significance level of 0.01 but no effect at 0.001, while no variation was detected in *Vp-1B* and *Vp-1D* (Tan et al. 2006). In this study, by using genomic-specific primers, two new *Viviparous-1* allelic variants related to PHS tolerance were explored on 3BL chromosome of bread wheat. Compared with *Vp-1Ba*, the new alleles *Vp-1Bb* had a 193-bp insertion at the position 2497-bp of AJ400713, which was located inside the third intron, and *Vp-1Bc* had an 83 bp deletion occurring at the position of 2,712 bp in the same intron region. The insertion itself had several exon–intron boundary of GT-AG, while the deletion had exactly the characteristics starting at 5′-GC and ending at 3′-AG and might act as an intron (Fig. 2) (Yan et al. 2000; Alexei et al. 2003). The reason for these observed alternative slicing forms is not known but may be related to the potential intron–exon boundary sequences, which are not efficiently spliced (Wilkinson et al. 2005). Furthermore, the insertion and deletion may affect stability of pre-mRNA structure and the expression level of correctly spliced *Vp-1* transcripts as observed in this study, with more correctly spliced *Vp-1B* transcripts in 35 DAP mature embryos present in genotype with *Vp-1Bb* than that with *Vp-1Ba* (Fig. 3), showing a close correlation with the degree of seed dormancy as observed by Nakamura and Toyama (2001). Moreover, it was also observed that most of the

Table 3 Occurrence of the three PCR fragments amplified with *Vp1B3* in 89 white-grained cultivars and landraces

PHS tolerance	Total	Average of GI × 100	Range of GI × 100	845 bp	569 bp	652 bp
Tolerant cultivars	39	7	0–26	4 (10%)	31(80%)	4 (10%)
Intermediate cultivars	30	42	24–70	5 (17%)	7 (23%)	18 (60%)
Susceptible cultivars	20	90	61–100	0 (0%)	2 (10%)	18 (90%)

PHS tolerant genotypes had either *Vp-1Bb* or *Vp-1Bc* genotypes. Majority of the genotypes with the 193 bp insertion were PHS tolerant landraces with germination rates below 8%, for example, Yibingbaimai, Yongchuanbaimai, Wanxianbaimai, Langzhongbaimai and Fulingbaimai. These landraces could therefore be used as useful genetic resources to develop PHS tolerant wheat cultivars.

In this study, 89 white-grained cultivars were used to validate this newly developed *Vp1B3* marker. Frequency of 569-bp fragments is 80% in PHS tolerance cultivars and landraces, while that of 652-bp fragments is 90% in susceptible cultivars (Table 3). Furthermore, statistical analysis indicated that *Vp1B3* was strongly associated with PHS tolerance in this set of Chinese germplasm, suggesting that *Vp1B3* could be used as an efficient and reliable co-dominant marker for the evaluation of wheat germplasm for PHS tolerance and marker-assisted breeding for PHS tolerant cultivars. However, although most of the genotypes with amplified 569-bp fragments were tolerant to PHS, some were susceptible. Moreover, four cultivars with 652-bp fragments showed PHS tolerance phenotype (Table 3). This may be due to the presence of many other factors in addition to seed dormancy affecting PHS. For instance, GA-oxidase, coat-imposed dormancy, spikes characters affecting penetration of water, and environmental factors (McCarty et al. 1991; Bailey et al. 1999; Li et al. 2004). In addition, our further study also indicated that this *Vp1B3* STS marker showed polymorphism among red-grained wheats differing in PHS tolerance, however, it was more closely linked to PHS tolerance in white-grained wheat genotypes than in red-grained ones (unpublished data). Red grain color of wheat (controlled by *R* genes) is known to be associated with the development of grain dormancy (Flintham 2000; Warner et al. 2000; Himi et al. 2002). *R* genes are transcriptional activators of the flavonoid synthesis genes (Himi and Noda 2005), which are located on the long arm of chromosomes 3A, 3B and 3D, approximately 30 cM proximal to the *Vp-1* locus, consistent with the observation of linkage between grain dormancy and red grain (Gale and Lenton 1987; Groos et al. 2002; Himi and Noda 2005). Although it is now clear that red color per se is not sufficient to guarantee dormancy (Flintham et al. 1999), combining dominant R alleles (R-A1, R-B1 and R-D1) at 2 or 3 loci would confer an additive effect on dormancy (Flintham 1993). Therefore, the opportunities for improving PHS tolerance may come true by combining the coat-imposed dormancy associated with red seed color and true embryo dormancy regulated by *Vp-1* (Baily et al. 1999).

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