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Multiplex PCR Identification of 1BL·1RS Translocation and High Molecular Weight Glutenin Allele *Glu-D1d* in Wheat

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Abstract: Wheat bread-making quality is greatly affected by high molecular weight glutenin subunits (HMW-GS) and 1BL·1RS translocation. Of the glutenins, subunits 1Dx5+1Dy10 encoded by the *Glu-D1d* gene have the largest positive effect on dough strength. And serious defects in bread quality have been associated with the presence of 1BL·1RS translocation. Therefore, it has been important to incorporate the *Glu-D1d* gene into bread wheat and simultaneously avoid the 1BL·1RS translocation. In this paper, a multiplex-PCR, composed of two sets of co-dominant markers, was developed to identify the *Glu-D1d* gene and the 1BL·1RS translocation. With the advantage that the multiplex PCR could simultaneously distinguish homozygous genotype from heterozygous at both loci, it's very useful and efficient in molecular assistant selection (MAS) breeding. It was successfully applied to scan a small size segregating F_2 population, and the results were consistent with that of the protein electrophoresis (SDS-PAGE). Furthermore, the multiplex PCR was validated in a collection from all the ten wheat zones in China and abroad by comparing simultaneously with the PCR assays using the markers of single locus.

Key words: Wheat; *Glu-D1d* gene; 1BL·1RS translocation; multiplex PCR

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利用多重 PCR 技术鉴定小麦背景中的 1BL·1RS 易位和 *Glu-D1d* 基因*

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摘　要:高分子量谷蛋白亚基(HMW-GS)对小麦面粉加工品质有促进作用,尤其是 *Glu-D1d* 基因编码的 1Dx5+1Dy10 亚基能增加面团的筋度和弹性。小麦背景中的 1BL·1RS 易位对小麦面粉加工品质有显著的负面影响。因此,在小麦品质育种中如何判定小麦背景中是否含有 1BL·1RS 易位和 HMW-GS 的 *Glu-D1d* 基因具有重要意义。本研究利用 3 对分别检测 1BL·1RS 易位、*Glu-B3* 和 *Glu-D1* 位点的共显性特异标记,结合 SDS-PAGE 鉴定,对 16 份已知遗传背景和 *Glu-D1x* 等位基因材料及 38 株(周麦 18×烟农 19)F_2 群体进行了分析,探索出适合同时鉴定小麦背景中 1BL·1RS 易位和 *Glu-D1d* 基因的多重 PCR 技术实验体系,并采用该体系对国内外 352 份小麦品种(系)进行了鉴定。结果表明,该体系是同时鉴定小麦背景中 1BL·1RS 易位和 *Glu-D1d* 基因的一种非常有效、简便可行的实验方法,可在标记辅助选择(MAS)育种中应用。

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关键词：小麦；1BL·1RS 易位；*Glu-D1d* 基因；多重 PCR

The quantity and quality of the proteins in the endosperm are major determinants of wheat end-use quality. High-molecular-weight (HMW) glutenin subunits, controlled by the complex *Glu-1* loci on the long arms of 1A, 1B, and 1D chromosomes, are mainly responsible for the viscoelastic properties of dough [1]. Of the HMW glutenins, subunits 1Dx5＋1Dy10 encoded by *Glu-D1d* gene have been found to have the largest positive effect on dough strength, whereas the allelic subunits 1Dx2＋1Dy12 have a negative effect [1-4]. Unfortunately, owing to the reason of highlighting on improving productivity during the second half of the 20th century in Chinese wheat breeding programs, the frequency of HMW-GS 1Dx5＋1Dy10 in Chinese wheat varieties was very lower [5-7].

The 1BL·1RS wheat-rye translocation has been widely used in wheat breeding programs all over the world. It was originally selected for the resistance to multiple diseases and was later confirmed that 1RS carry genes conferring to leaf, stem and stripe rusts and powdery mildew, which had been designated as *Lr*26, *Sr*31, *Yr*9 and *Pm*8. Besides the disease resistance, the lines with 1BL·1RS translocation performed very well in high yielding, yield stability and wide adaptation. So, in spite of the loss of resistance, which caused by the change of prevalent physiological race of pathogen, the translocation was still widely used in many breeding programs. In China, more than one third of varieties carry the translocation both in the predominant cultivars of recent 30 years [8] and in newly developed wheat germplasm [9]. However, serious defects in bread quality such as poor mixing tolerance, low SDS-sedimentation volume, dough stickiness, and low bread volume have been reported with the presence of the translocation [10-12].

It was reported that high frequency of undesirable HMW-GS and LMW-GS, as well as the presence of the 1BL·1RS translocation are responsible for the weak gluten property of Chinese germplasm [13]. In addition, the presence of HMW 1Dx2＋1Dy12 glutenin subunits and 1BL·1RS translocation produces a drastic decline in gluten strength, making it difficult to find acceptable quality among genotypes possessing both negative factors [14]. So, avoiding the 1BL·1RS translocation and integration of desirable subunits at *Glu-1* such as HMW 1Dx5＋1Dy10 could lead to the improvement of gluten quality in Chinese wheat.

Traditionally, the composition of HMW-GS and 1BL·1RS translocation was identified by SDS-PAGE or A-PAGE analyses. Many research works have been conducted on the Chinese bread wheat varieties for the HMW-GS composition and 1BL·1RS translocation [5, 8-9, 13] by the protein electrophoresis method. Though it is a standard and effective method in detecting variations in HMW-GS and 1BL·1RS translocation, SDS-PAGE and A-PAGE is not suited for high throughput screening in breeding programs because it is time consuming and laborious. In addition, the materials in SDS-PAGE or A-PAGE analyses are limited in mature grains, which make the identification after the field selection. Therefore, functional molecular markers, which based on the gene sequences, have been developed for the selection of glutenin subunits 1Dx5＋1Dy10 [15-19] and for the detection of the presence of 1BL·1RS translocation [20-23], respectively, targeting at providing accurate and high throughput data for determination of alleles in breeding materials. Furthermore, several multiplex PCR [17, 24-27], involved in wheat quality properties, have been designed, with the advantage of reducing costs and increasing efficiency of marker-assisted selection. But none of the above multiplex PCR was co-dominant for all the loci detected. So, it could not provide more detail genetic information on the

locus assayed, and was not perfect for the wheat breeders. Here, a multiplex PCR, based on two sets of co-dominant markers [19, 22], was reported, which could identify homozygous and heterozygous genotype both at the *Glu-D1d* gene and 1BL · 1RS translocation locus.

1 Materials and Methods

1.1 Plant materials

Sixteen varieties (lines) were selected for optimizing the reaction condition of the multiplex PCR. In these cultivars, there were three alleles in *Glu-D1x* locus, encoding the subunits of 1Dx2, 1Dx4 and 1Dx5, respectively. And a F_2 population was used in validation of the multiplex PCR, which was derived from a cross between Zhoumai18 (with *Glu-D1a* gene and 1BL · 1RS translocation) and Yannong19 (with *Glu-D1d* gene and the normal 1B chromosome).

A total of 352 accessions, including 220 modern varieties and 63 landraces from the ten wheat production zones in China, 66 materials introduced from abroad, and three synthetic wheat lines from the cross of *Triticum carthlicum* with *Aegilops tauschii*, was used to validate the stability and reliability of the multiplex PCR. At the same time, PCR assays with the two single locus co-dominant markers were conducted to reconfirm the multiplex PCR. Of the 220 modern varieties, 146 varieties were bred after 1970, when the 1BL · 1RS translocation lines (such as Lovrin 10, Lovrin 13, Predgornia 2, Aurora, Kavkaz and Neuzucht *et al.*) were introduced into China for the resistance to strip rust (caused by *Puccinia striiformis* West.) and powder mildew (caused by *Blumeria graminis*). Most of the 66 foreign materials (38 accessions) were from Europe, and 10, 7, 7 and 4 of them from North America, South America, Australia and East Asia, respectively.

1.2 Multiplex PCR analysis

The genomic DNA of the sixteen varieties (lines) was extracted from leaves of seedling according to the method of Devos [28]. For the F_2 population, kernels were split into two half: the embryo-end was germinated to produce seedling, where DNA was extracted from seedling and coleoptile following Chai [29]; the other end was ground and used for glutenin protein extracts (see SDS-PAGE analysis section). The primers were synthesized from Invitrogen Corporation (http://www.invitrogen.com.cn) on the basis of the data accessible in literature (Table 1). PCR reactions were performed in a Biometra T1 Thermal Cycler (Whatman Biometra, Inc.) with a heated lid in the final volume of 20 μL. The PCR reaction mixture contained 1 × buffer (Takara), 2.25 mM $MgCl_2$, 250 μM of each dNTP, 0.36/0.36/0.24/0.24/ 0.14/0.07/0.21 μM primer of Sec-P1/Sec-P2/Glu-B3-F/Glu-B3-R/Dx-F/Dx5-F/Dx-R, 125 ng genomic DNA and 1.2 units Taq DNA polymerase (Takara). After the initial temperature of 95°C for 10 min, 35 cycles of 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C were performed, followed with an additional 10 min extension.

Table 1 Primer sequences and expected sizes of PCR fragment

Gene	Primer	Sequence(5′……3′)	Size of fragment (bp)	Reference
Sec-1	Sec-P1	ACCTTCCTCATCTTTGTCCT	1 076	[22]
	Sec-P2	CCGATGCCTATACCACTACT		
Glu-B3	Glu-B3-F	GGTACCAACAACAACAACCC	636	[22]
	Glu-B3-R	GTTGCTGCTGAGGTTGGTTC		
Glu-D1d	Dx-F	TTTGGGGAATACCTGCACTACTAAAAAGGT	343/361*	[19]
	Dx5-F	AAAAGGTATTACCCAAGTGTAACTTGTCCG		
	Dx-R	AATTGTCCTGGCTGCAGCTGCGA		

* The size of PCR products for *Glu-D1d* and other alleles, respectively.

1.3 SDS-PAGE analysis

Glutenin protein extracts were prepared according to Liu *et al*[13] and Singh [30] with small modifications: a ground whole kernel of the sixteen varieties or the half kernel (distal embryo-end) of the F_2 population was extracted with 1.5 mL of 50% propanol (v/v) for 5 min under continuous vortex mixing, followed by incubation (20 min at 65°C), vortexing (5 min), and centrifugation (5 min at 6 600 g). The supernatant and residue obtained were considered as the final gliadin plus secalin (when 1BL·1RS translocation was present) and glutenin extracts, respectively. These two fractions were separately prepared for SDS-PAGE analysis according to Singh *et al*[30]. HMW-GS were classified using the nomenclature of Payne and Lawrence [31] and the LMW-GS according to the nomenclature of Singh *et al* [30] and Jackson *et al*[32]. The presence of *Glu-B3j* (null allele) and *ω-secalin* indicated the presence of the 1BL·1RS translocation [33]. A 13% SDS-polyacrylamide gel was used to separate gliadin and HMW glutenin subunit extracts.

2 Results

2.1 Optimization and validation of the multiplex PCR

The multiplex PCR was composed of seven primers: two primers were specific for *Sec-1* gene, produced a 1 076 bp fragment in size [27]; two primers were specific for *Glu-B3* locus, gave an expected fragment of 636 bp in size [27]; the other three primers could generate a 343-bp or 361-bp specific PCR product for the *Glu-D1d* gene and other alleles on the Glu-D1x locus, respectively [19]. After a series of experimentation on the concentration of $MgCl_2$, dNTP, primers, and also on the proportion between the primers, an ideal multiplex PCR reaction condition was created.

The multiplex PCR results for 16 varieties (lines) were shown in Fig. 1 (upper). The 343-bp fragment produced in the lanes of 1, 4, 5, 6, 8, 10, 12, 13 and 14 indicated the presence of HMW 1Dx5 subunit; the 361-bp fragment amplified in the lanes of 2, 3, 7, 9, 11, 15 and 16, indicated the absence of HMW 1Dx5 subunit. This result was in accordance with the SDS-PAGE analysis (Fig. 1 middle). The specific primers for *Sec-1* gene amplified a 1 076 bp fragment in lanes of 3, 4, 7, 8, 10, 11, 15 and 16, which implied that these varieties (lines) were 1BL·1RS translocation; the *Glu-B3* primers amplified a 636-bp fragment from lanes 1, 2, 5, 6, 9, 12, 13 and 14, which inferred that these varieties had normal 1B chromosome and without 1BL·1RS translocation. And the SDS-PAGE analysis of LMW-GS and *ω-secalin* protein confirmed those results simultaneously (Fig. 1 lower): *ω-secalin* protein and the *Glu-B3j* allele existed in the lanes of 3, 4, 7, 8, 10, 11, 15 and 16; but in the other lanes, *ω-secalin* protein was absent and other alleles of *Glu-B3* locus were present (*Glu-B3b* allele in 1, 9, 13 and 14; *Glu-B3f* allele in 2; *Glu-B3h* allele in 5 and 6; *Glu-B3d* allele in 12).

The DNA of couple of varieties (Chinese Spring and Predgornia 2; Zhoumai18 and Yannong19), which both have different HMW glutenin subunits in *Glu-1D* locus and disagree on 1BL·1RS translocation each other, was mixed respectively to make two heterozygous DNA plates artificially (lane 17 and lane 18). As shown in Fig. 1, all the amplicons (1 076-bp, 636-bp, 361-bp and 343-bp) were produced simultaneously in the multiplex PCR reaction, when the mixed DNA was used as PCR templates. Therefore, the multiplex PCR was co-dominant both for the two loci detected, and could identify the heterozygous genotype of *Glu-1D* locus and 1BL·1RS translocation at the same time.

2.2 Application of the multiplex PCR in a segregating F_2 population

The multiplex PCR analysis was performed on 38 randomly selected F_2 plants from the cross Zhoumai 18 (1BL·1RS translocation with HMW-GS 1Dx2+1Dy12) and Yannong19 (non-1BL·1RS with HMW-GS 1Dx 5+1Dy 10).

And the composition of HMW-GS, allele variation of Glu-B3 and the presence of *ω-secalin* pro-

M: 100-bp ladder marker; 1: Zhongyou9507; 2: Chinese Spring; 3: Lovrin 10, 4: Predgornia 2; 5: Mara; 6: Yannong19; 7: Lianmai9791; 8: Lankao906; 9: Qinnong42, 10: Huaimai18; 11: Zhoumai18; 12: Zheng3666; 13: Nannong04B1258; 14: Yanshi02-1; 15: Shimai14; 16: Shimai4185; 17: Chinese Spring+Predgornia 2; 18: Zhoumai18+Yannong19.

Fig. 1 PCR products derived from the multiplex PCR using *Sec-1*, *Glu-B3* and *Glu-D1d* genes specific primers (uper); the SDS-PAGE patterns of HMW glutenin subunits (middle); the SDS-PAGE patterns of LMW glutenin subunits and ω-secalin protein (lower).

tein of the F_2 plants was identified simultaneously by the SDS-PAGE methods (Fig. 2). The results from the multiplex PCR were absolutely in agreement with those obtained by SDS-PAGE analysis. Homozygous and heterozygous F_2 plants, both in the *Glu-1D* and 1BL·1RS translocation locus, were easily distinguished. Of the 38 F_2 plants, only one was homozygous 1B chromosome and homozygous *Glu-D1d*, which was the ideal genotype for the pan bread quality and did not need further genotyping in its offspring on these two loci; five plants were heterozygous both at the two loci; five and 12 plants were homozygous in 1BL·1RS translocations and homozygous *Glu-D1a* respectively, both of them had negative influence on bread-making quality and should be discarded in the pan bread wheat breeding program; ten plants were homozygous in 1B chromosome and heterozygous in *Glu-D1d*; five plants were homozygous *Glu-D1d* and heterozygous 1BL·1RS translocations. In the last two cases, the offspring of these plants just

M: 100-bp ladder marker; P1: Zhoumai18 (*Glu-B1j*); P2: Yannong19 (*Glu-B1h*); 1~19 and 20~38: F_2 plants derived from the cross Zhoumai18/Yannong19.

Fig. 2 PCR assay with the multiplex PCR (uper), SDS-PAGE assay for the HMW glutenin subunits (middle) and SDS-PAGE assay for LMW glutenin subunits and *ω-secalin* protein (lower) in a small F_2 population

need to be detected on one locus, and only one of the co-dominant PCR markers reported previously [19, 22] is needed correspondingly.

2.3 Application of the multiplex PCR in a germplasm collection

The result detected with the multiplex PCR in the 352 accessions was completely uniform with that of the two single locus co-dominant markers. And the multiplex PCR performed stable in all the reactions and replications. Eight genotypic compositions of the two loci were found in the germplasm collection (Table 2). A small part of the collection (3.41%) was found heterozygous either on the 1BL・1RS (7 accessions) or *Glu-D1d* gene locus (5 accessions), but none of the materials was heterozygous for both two loci. Fifty-five (15.63%) and 51 (14.49%) accessions were homozygous in 1BL・1RS and *Glu-D1d* gene respectively, including 11 varieties which was both homozygous on the two loci. The genotype absent in both 1BL・1RS translocation and *Glu-D1d* gene, was the predominant genotype and hold 70.74% in the collection.

Seventeen accessions of 66 foreign varieties (25.76%) had *Glu-D1d* gene. On the contrary, only one landrace from China (1.59%) had *Glu-D1d* gene. And a lower frequency of *Glu-D1d* gene (14.09%) was found in modern varieties from China, which agree with the result of He (16.8%; 1992), Liu (20.4%; 2007), and Zhang (18.59%; 2002). For the 1BL・1RS locus, the frequency of 1BL・1RS translocation in 146 modern varieties, bred after 1970 in China, was 40.41%, which was very similar with the report of Zhou [8]. Eighteen varieties (12.32%) was detected in the 146 China modern varieties, which had *Glu-D1d* gene and absent for 1BL・1RS translocation.

Table 2 Distribution of genotype on the loci of *Glu-1D* and 1BL・1RS in the 352 accessions

Glu-1D	1BL・1RS	Modern varieties	Landraces	Foreign materials	Synthetic wheat	Total	Percent /%
−	−	138	62	46	3	249	70.74
−	+	40		2		42	11.93
+	−	21	1	16		38	10.80
+	+	10		1		11	3.13
−	H	5				5	1.42
+	H	2				2	0.57
H	−	2		1		3	0.85
H	+	2				2	0.57
Total		220	63	66	3	352	100

+/−: the homozygous presence/absence of *Glu−D1d* or 1B・1R; H, heterozygous genotype on the two loci.

3 Discussion

In recent years, considerable emphasis has been placed on the marker-assisted selection (MAS) in plant breeding[34-35]. Functional markers (FMs, also called perfect or diagnostic markers) have been considered as the ideal tools for MAS breeding[36-37]. With the great progress of gene cloning, more and more FMs, derived from polymorphic sites within genes that causally affect phenotypic trait variation, have been developed [37]. Many FMs related on bread-making quality (such as HMW-GS, LMW-GS, waxy protein, ω-secalin protein and grain texture) were reported and applied in wheat breeding [18, 23-24, 26, 38-40]. Co-dominant functional molecular marker has more advantage than common FMs in regard to giving complete genetic information on the locus detected, which is very useful in wheat breeding. Since the objection of wheat breeding is to create a pure line with homozygous loci, of course, the dominant marker cannot offer the information directly. To verify

whether the locus of a line is homozygous, a dominant marker has to be applied on the population derived from the line, and only the segregation of locus on the population can give a clear answer. In this paper, a multiplex PCR was put forward based on two co-dominant markers reported previously [19, 22]. And as the result has shown, it could easily recognize the homozygous genotype both on the two loci.

The frequency of *Glu-D1d* in Chinese bread wheat was no more than 20% [5-7], and the frequency of 1BL • 1RS translocation varieties was nearly about 40%. So, the ratio of crosses, involved both the negative factors of non-*Glu-D1d* and 1BL • 1RS translocation, was about 32% (80% * 40%). Analysis and genotype selection both on the two loci was needed in these crosses in pan bread wheat breeding programs. The use of multiplex PCR reported had been successfully applied in MAS breeding in such situation.

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野生二粒小麦抗条锈病基因*YrH52*的 RGA 分子标记

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摘 要:为开发与野生二粒小麦抗条锈病基因 *YrH52*紧密连锁的分子标记,并为该基因的克隆及应用奠定基础,运用 RGA (Resistance gene analog) 分子标记法,以*YrH52*定位作图 F_2 群体形成的 F_4 抗性和感病基因池(Gene pool)及其亲本(抗病材料 H52 与感病材料 Ldn)进行多态性筛选分析,共获得 17 个 RGA 分子标记。使用已有的遗传图并进行 MultiPoint 分析,构建了由与抗性(H)和感病(L) 两个亲本对应的显性位点组成的两个遗传图,即 H 遗传图和 L 遗传图。在 H 图中,*YrH52*与 10 个 RGA 标记,即*X_uhw3*, *X_uhw17*, *X_uhw18*, *X_uhw23*, *X_uhw36*, *X_uhw38*, *X_uhw46*, *X_uhw59*, *X_uhw62* 和*X_uhw73* 紧密连锁,其中*X_uhw23* 标记为共显性分子标记,连锁距离为 1.0 cM。在 L 图中,发现*X_uhw57*, *X_uhw68*, *X_uhw189*, *X_uhw192* 及其*X_uhw23* 与*YrH52* 相聚成簇(Cluster)。本研究结果说明 RGA 分子标记结合集群分离分析法(Bulked segregant analysis,BSA)是一种快速开发与小麦抗病基因紧密连锁标记的有效方法,对小麦抗条锈病分子育种和抗病基因的克隆具有促进作用。

关键词:野生二粒小麦;条锈病基因 *YrH52* ;遗传图;抗病基因类似序列;分子标记

中图分类号:S512.1;S336 **文献标识码:**A **文章编号:**1009-1041(2011)04-0590-08

Resistance Gene Analog Markers Linked to the Stripe Rust Resistance Gene *YrH52* Derived from Wild Emmer Wheat, *Triticum dicoccoides*

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Abstract: The wheat stripe rust resistance gene *YrH52*, derived from wild emmer wheat, *Triticum dicoccoides*, was previously mapped on chromosome 1BS. The aim of the present study was to develop resistance gene analog (RGA) markers linked to *YrH52* for future marker-assisted selection in wheat and cloning of the resistance gene *YrH52*. The bulked segregate analysis (BSA) was used to screen DNA pools of resistant vs. susceptible progenies, and 17 RGA markers linked to *YrH52* were obtained. Two versions of genetic maps were constructed with skeleton and added markers by MultiPoint based on the previous linkage map of *T. dicoccoides*, respectively. As added markers in the H version map, the *YrH52* and 10 RGA markers (*X_uhw3*, *X_uhw17*, *X_uhw18*, *X_uhw23*, *X_uhw36*, *X_uhw38*, *X_uhw46*, *X_uhw59*, *X_uhw62* and *X_uhw73*) were linked tightly together within genetic distance of 1.0 cM. In the L version map, four RGA markers, *X_uhw57*, *X_uhw68*, *X_uhw189*, *X_uhw192* and *also X_uhw23* were clustered with *YrH52*. These results demonstrate the RGA sequences

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