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Research note

Development of functional marker for distinguishing *Glu-B3b* allele of LMW-GS found in Indian common wheat cultivars

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LMW glutenins constitute around 40% of the total proteins in wheat grain and contribute significantly in imparting gluten strength and extensibility. However, it is difficult to have unambiguous classification of LMW glutenins by SDS-PAGE (Ikeda et al., 2008) because of their large numbers and overlapping mobility with gliadins in the gel. Recently, PCR based markers have been developed (Liu et al., 2012; Sui et al., 2010; Wang et al., 2009; Zhang et al., 2011) for identification of LMW glutenin alleles. However, some of these markers could not distinguish LMW glutenin alleles in diverse Indian cultivars (Ram et al., 2011). For example *Glu-B3b* allele could not be identified by the primer sets (SB2F/SB2R and SB10F/SB10R) developed by Wang et al. (2009) in Indian cultivars; there was amplification in 21 out of 51 cultivars having *Glu-B3b* allele. *Glu-B3b* and *Glu-B3g* have significant positive effects on gluten strength (Liang et al., 2010; Maucher et al., 2009; Tabiki et al., 2006) and are present in 60% of Indian wheat cultivars (Ram et al., 2011). Development of markers distinguishing these alleles has relevance in breeding for improving wheat quality. To understand the reasons for disparity in PCR and SDS-PAGE and to develop a *Glu-B3b* specific primer, two of the LMW glutenin gene sequences (EU369719 and EU369721) reported by Wang et al. (2009) representing the *Glu-B3* locus were cloned and sequenced from Indian wheat cultivars. The nucleotide sequences of EU369719 and EU369721 correspond to LMW-m and LMW-s types, respectively.

Genomic DNA was isolated as per the modified method of Benito et al. (1993). PCR amplification was performed in a total volume of 20 μl containing 50–100 ng of genomic DNA, 1× PCR buffer, 1.5 mM of $MgCl_2$, 200 μM of each deoxyribonucleotide (dNTP), 100 ng of each primer and 0.3 U of Taq DNA polymerase (Bangalore Genei). PCR products of both the genes were separated on agarose gel, excised, purified and cloned into pJET1.2 cloning vector (CloneJet™). The sequencing work was performed by Chromous Biotech India. NCBI BLAST and ClustalW were used for sequence analysis. The phylogenetic tree was constructed using *MEGA* 4.1.

The 281 base pair fragment comprising the 5′ flanking region of the EU369719 gene, which contained the binding site of the earlier designed forward primer for *Glu-B3b* (SB2F), was cloned from 8 wheat cultivars possessing the *Glu-B3b* allele using primer set LB4F/IP4R. The primer combination SB2F/SB2R showed amplification in 4 of the cultivars, namely HD 2501, HI 1500, K 7903 and NP 818 and no amplification in HD 1941, HD 1981, PBW 396 and UP 215. The sequence analysis of the 5′ flanking region of EU369719 from 8 cultivars showed 100% similarity among themselves and differed from the sequence reported by Wang et al. (2009) in one of the nucleotides in the forward primer binding site (G to A) (Fig. A1).

Abbreviations: LMW-GS, low molecular weight glutenin subunits; PCR, polymerase chain reaction; SDS-PAGE, sodium-dodecyl-sulphate polyacrylamide gel electrophoresis.

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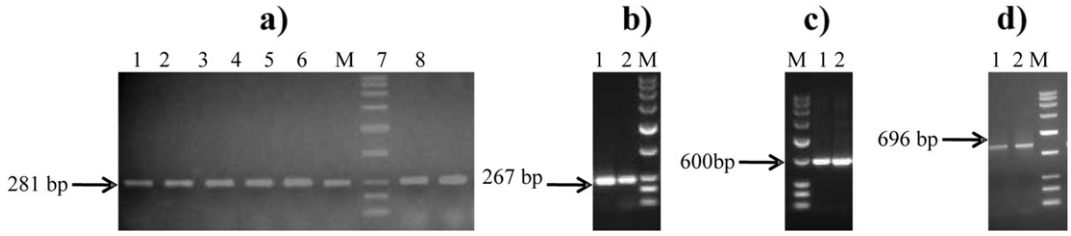

Fig. 1. Agarose gel electrophoresis. **a**; 281-bp PCR amplification product of 5′ flanking region of EU369719 using the LB4F/IP4R primer pair in a set of Indian wheat cultivars. 1 Lal Bahadur, 2 PBW 396, 3 UP 215, 4 WH 283, 5 UP 368, 6 K 7903, 7 WH 157, 8 NP 818, M DNA ladder. **b**, **c** and **d**; PCR amplification product of three fragments representing full length *Glu-B3b* genes using primer pairs LB2F/IP1R, IP2F/IP2R and IP3F/LB2R, respectively. 1 HD 1925, 2 HD 2270.

There was no difference in sequences of the binding site for SB2F in the 5′ flanking region of all the cultivars and all other alleles encoded by the same locus. In addition to SB2F/SB2R, several other internal forward primers were designed based on the EU369719 sequence to distinguish *Glu-B3b* allele in combination with reverse primer (SB2R). However, these primer combinations could not distinguish the *Glu-B3b* allele.

Therefore, the full length EU369721 gene was cloned and sequenced from two Indian cultivars having *Glu-B3b* allele and one of them, namely HD 1925, showed amplification while HD 2270 did not amplify with SB10F/SB10R. There was a problem in cloning the full length gene at a stretch and hence the gene was sequenced in three overlapping fragments of the size of 267, 600 and 696 base pair length from 5′ to 3′ direction (Fig. 1). Primer sequence (5′-3′) designed and used in amplification of fragments representing the *Glu-B3b* gene and their location in reference genes are listed in Table 1. The sequences of three fragments were aligned to identify overlapping sequences and consequently full length gene sequences were obtained from HD 1925 and HD 2270 cultivars and designated as LMWB3.1 and LMWB3.2 (GenBank accessions JX163861 and JX163862), respectively. Multiple alignments (Clustal W) of both the sequences showed 99.5% identity between them and 99% with EU369721 (Fig. A2).

Table 1
The list of primer sequences designed for amplification and cloning of the 5′ flanking region and full length sequences of *Glu-B3b* based on the reference genes EU369719 and EU369721, respectively, their location and PCR conditions. IP1F and IP3R correspond to LB2F and LB2R of Wang et al. (2009), respectively. The primer location is given based on the distance from the first base of the start codon of reference genes.

Primer name	Sequence (5′-3′)	Primer location	PCR conditions
Primers for amplification of the flanking region of *Glu-B3b* (EU369719)			
LB4F	CACCTATACAAGGTTCCAAAAT	−448	94 °C/3 min; 94 °C/30 s −58 °C/30 s
IP4R	TAGGTTGCACTAGCTATA	−167	72 °C/45 s, 35 cycles; 72 °C/10 min
Primers for amplification of 3 fragments representing the full length sequence of *Glu-B3b* (EU369721)			
IP1F	AACCTAACGCATTGTACCAAAAATC	−173	94 °C/3 min; 94 °C/30 s −58 °C/30 s
IP1R	GTCTCTCCAAACCAGGGATG	94	72 °C/45 s, 35 cycles; 72 °C/10 min
IP2F	CCATGAAGACCTTCCTCATCTT	−2	94 °C/3 min; 94 °C/30 s −59 °C/30 s
IP2R	TGCAAGATAGATGGATGAACAAA	598	72 °C/60 s, 35 cycles; 72 °C/10 min
IP3F	CAACCAAGTCTACCGCAACAAC	522	94 °C/3 min; 94 °C/30 s −57 °C/30 s
IP3R	GGCGGGTCACACATGACA	1220	72 °C/60 s, 35 cycles; 72 °C/10 min

When compared to EU369721, the coding region of LMWB3.1 and LMWB3.2 showed 8 and 10 SNPs, respectively. Among them, there were 5 synonymous mutations in LMWB3.1 and 6 in LMWB3.2. In both the genes, there was C-G substitution at the 134th position leading to an amino acid change from serine to tryptophan, T−C transition at the 181th position leading to amino acid change from serine to proline and C-A base change at the 407th position leading to amino acid mutation from proline to glutamine in the repetitive domain. There were synonymous mutations at 3 positions between LMWB3.1 and LMWB3.2 sequences and one A-G base substitution at the 637th position leading to amino acid change from lysine to glutamic acid in the C-terminal domain of the deduced peptide of LMWB3.2. Since there was no change in the length of the repetitive domain and number and position of cysteine residues in *Glu-B3b* in Indian wheat cultivars as compared to the known sequence, no functional difference is expected. The sequence of the gene in both the cultivars at the forward primer (SB10F) binding site was similar to *Glu-B3g* and differed from the sequences of all other alleles encoded by the same locus in 1–5 nucleotide positions (Fig. 2**a**). Since Wang et al. (2009) used mismatched nucleotides in 2 positions in designing the forward primer (SB10F), there were differences at 3 positions when compared to the sequences in Indian cultivars. Moreover, the complementary sequence of the reverse primer (SB10R) was similar in all other alleles encoded by the same locus. Therefore, the primer pair (SB10F/SB10R) showed inconsistency in differentiating the *Glu-B3b* allele.

The full length sequence of the gene in this investigation was used in designing several combinations of primers for distinguishing the *Glu-B3b* allele. One of the combinations had forward primer with sequence similarity between both the cultivars as well as between *Glu-B3b* and *Glu-B3g* alleles, but differed from other alleles in 1–5 nucleotide positions at the primer binding site (Fig. 2**a**). The reverse primer was designed such that there was a difference of one nucleotide between *Glu-B3b* and *Glu-B3g* alleles while 2–3 nucleotide differences with other alleles encoded by the same locus (Fig. 2**b**). The forward and reverse primer sequences were S1F (5′-GCAACAACAACAAATAGTACTACAG-3′) and S1R (5′-CGGTGCCAACACCGAATGGCACAT-3′), respectively. The amplification conditions were 94 °C/3 min; 94 °C/30 s–63 °C/30 s–72 °C/45 s, 35 cycles; 72 °C/10 min. The size of the amplified product was of 610 bp (Fig. 3).

The marker developed in this investigation was used in the identification of *Glu-B3b* in a set of 182 diverse bread wheat cultivars developed in India, carrying different *Glu-B3* alleles as determined by SDS-PAGE (Ram et al., 2011). There was amplification in all of the 51 cultivars having *Glu-B3b* and 47 with *Glu-B3g* alleles