



Molecular characterization of the *Puroindoline a-D1b* allele and development of an STS marker in wheat (*Triticum aestivum* L.)

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ABSTRACT

Kernel hardness is mainly controlled by one major genetic locus on the short arm of chromosome 5D in bread wheat. Twelve Chinese and CIMMYT wheat cultivars were characterized for the deletion region of *Pina-D1b* genotype and developing a novel STS marker for this allele. PCR and SDS-PAGE were used to confirm the *Pina-D1b* genotype, and then 20 pairs of primers were designed to amplify the fragment including deletion region in *Pina-D1b* genotype by primer walking strategy. An STS marker *Pina-N* spanning deletion region in *Pina-D1b* was developed and sequencing results showed that all of 10 *Pina-D1b* genotypes uniformly possessed a 15,380 bp deletion in comparison with that of Chinese Spring with wild type. This study provided an alternative method to exam *Pina-D1b* by molecular marker and will accelerate identification of puroindoline alleles in bread wheat.

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Kernel texture is a leading quality characteristic of bread wheat (*Triticum aestivum* L.). The molecular basis of kernel texture results from the puroindoline genes at the *Hardness* (*Ha*) locus on chromosome 5DS, and includes *Puroindoline a* (*Pina-D1*) and *Puroindoline b* (*Pinb-D1*) (Bhave and Morris, 2008). Soft endosperm varieties possess the 'wild-type' puroindoline alleles (*Pina-D1a* and *Pinb-D1a*), whereas all wheat varieties with hard endosperm have been found to possess one or more mutations in *Pina* or *Pinb*.

Different mutations in *Pina* and *Pinb* can result in differences in kernel texture (Bhave and Morris, 2008; Morris and Bhave, 2008). The two most prevalent *hardness* haplotypes are *Pina-D1b/Pinb-D1a* and *Pina-D1a/Pinb-D1b*. Studies indicate that the 'Pina-null' (*Pina-D1b*) allele is harder than the *Pinb-D1b* allele (Chen et al., 2006; Morris and Massa, 2003), and may be less desirable for milling, steamed bread, pan bread, and Chinese noodle than the *Pinb-D1b* genotype (Chen et al., 2007). Consequently, it is of great practical value to identify the hard allele of each of these two prevalent haplotypes.

Analysis of *Pinb-D1b* is straightforward as PCR can generate full-length *Pinb* amplicon, which can then be sequenced, or SNP allele-specific primers can be used (Giroux and Morris, 1998). Analysis of *Pina-D1b* is not so straightforward. In many studies, the *Pina-D1b*

allele was assigned based on hard kernel phenotype and absence of a mutation in *Pinb-D1* and/or absence of PINA on polyacrylamide gels (Chen et al., 2006; Giroux et al., 2000; Morris and Massa, 2003). Neither of these results can unequivocally confirm the presence of *Pina-D1b*. Further, more than one mutation may confer the PINA null phenotype (Chen et al., 2006; Gazza et al., 2005).

Accordingly, the 2007 Supplement of the *Catalogue of Gene Symbols for Wheat* reserved the *Pina-D1b* allele as a specific 15,380-bp deletion in *Pina* based on the NCBI sequence (AB262660) of Takeuchi et al. (unpublished) beginning after nucleotide 23 in the coding sequence. Ragupathy and Cloutier (2008) characterized a BAC (EU835982) that encompassed *Pina-D1b*.

The aim of this study was to further characterize the deletion in additional genotypes with *Pina-D1b*, and to develop a robust PCR-based molecular marker system to substitute for the time-consuming SDS-PAGE technique.

Four Chinese wheat varieties (Chinese Spring, Yunong 202, Gaocheng 8901 and Shaan 253) and eight CIMMYT cultivars and advanced breeding lines (Mo299, Mo293, Mo215, Mo191, Mo181, Mo272, RDWG/MILAN, CMH82A.1294/2*KAUZ//MUNIA/CHTO/3/MILAN) were used for the characterization of *Pina-D1b* genotype and development of molecular markers.

Extractions of genomic DNA and Triton-soluble proteins were performed following the procedures described by Chen et al. (2006) and Morris and Massa (2003), respectively. Two pairs of primers, the first *Pina-D* spanning the coding region of *Pina*

Abbreviations: PCR, polymerase chain reaction; PINA, puroindoline a protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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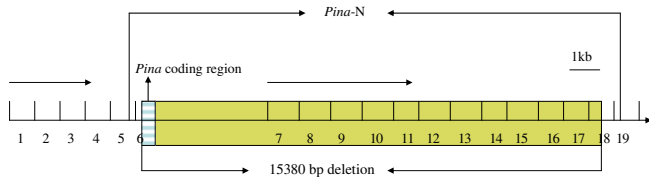


Fig. 1. Schematic diagram for the locations of 19 primers used for the amplification of *Pina-D1b* allele flanking the deletion regions.

(forward: CATCTATTTCATCTCCACCTGC; reverse: GTGACAGTTTATTAGTAGT) and Pinb-D spanning the coding region of *Pinb* (forward: GAGCCTCAACCCATCTATTTCATC; reverse: CAAGGGTGATTTTATTCATAG) were developed to further confirm the *Pina-D1b* genotype, and to check the DNA quality, respectively.

In order to obtain a fragment spanning the *Pina* deletion region, the first and 7th pairs of primers in Fig. 1 were developed from $-4,582$ bp to $-3,594$ bp of *Pina* 5' flanking sequence (ATG codon references zero) and $+4,188$ bp to $+5,107$ bp of *Pina* 3' flanking sequence, respectively, according to the *Ha* sequence in NCBI accession CT009735. The other 17 pairs of primers were designed by a primer walking strategy closer to or further away from the *Pina* coding region based on the success or failure of PCR amplification with previous primers. For example, the 2nd and 8th pairs of primers were designed based on the absence of PCR amplification with the 1st primer and presence of PCR amplification with 7th pair of primers, respectively. Finally, the forward (AATACCACATGGTTCTAGATACTG) and reverse (GCAATACAAAGGA CCTCTAGATT) primers of *Pina-N* were designed in the region with two fragments amplified with the 5th and 19th pair of primers in Fig. 1. These primers were expected to span the deletion present in *Pina-D1b* and thereby serve as diagnostic for this allele. All of the primers were designed by Primer Premier 5.0 software and sequence alignments were performed by DNAMAN software.

PCR amplifications were performed by following the procedure reported by Chen et al. (2006). PCR products were separated in 1.5% agarose gels and the relevant fragment was purified from the gels using Quick DNA extraction kit. The purified products were ligated into pGEM-T Easy vector and transformed into cells of *Escherichia coli* DH-5 α strain. Plasmids containing targeted fragments were extracted by Plasmid Rapid Isolation Kit and three clones for each sample were sequenced from both strands.

SDS-PAGE indicated that 10 varieties, including Gaocheng 8901 and Shaan 253, lacked PINA and therefore were putative *Pina-D1b* genotypes (Fig. 2). Primers *Pina-D*, which span the *Pina* coding region, and *Pinb-D*, which span the *Pinb* coding region, were used to check if the PINA null phenotype resulted from a deletion, i.e. *Pina-D1b*, since not all PINA null phenotypes may be *Pina-D1b*, according to previous reports (Chen et al., 2006; Gazza et al., 2005). Furthermore, absence of PCR products with *Pina-D* primer and presence of a 597-bp PCR amplification with *Pinb-D* primer suggested that all of these 10 varieties lacking PINA belonged to *Pina-D1b* genotype (Fig. 3).

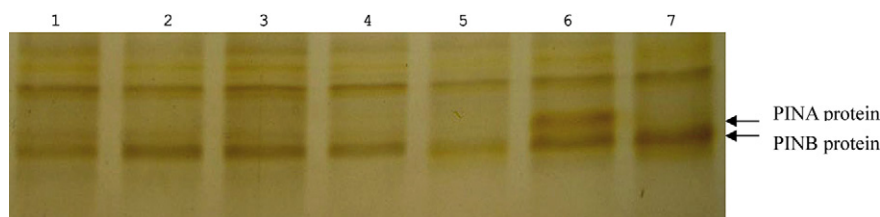


Fig. 2. SDS-PAGE of Triton X-114 extracted protein from single kernel to detect PINA null genotype in seven wheat cultivars. From left to right: Mo299 (Lane 1), M0191 (Lane 2), M0181 (Lane 3), RDWG/MILAN (Lane 4), Gaocheng 8901 (Lane 5), Chinese Spring (Lane 6) and Shaan 253 (Lane 7). Two arrows indicated PINA and PINB protein, respectively.

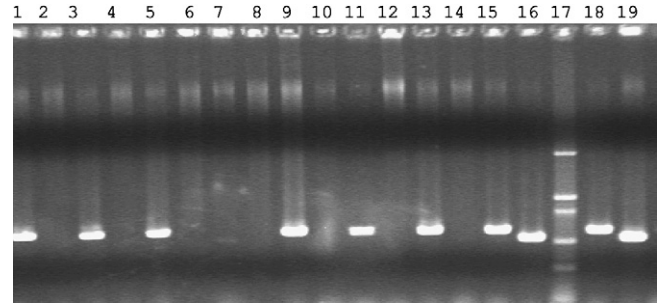


Fig. 3. PCR amplification with primers *Pina-D* and *Pinb-D* spanning *Pina* and *Pinb* coding regions. From left to right: Shaan 253 (Lanes 1 and 2), Gaocheng 8901 (Lanes 3 and 4), Mo299 (Lanes 5 and 6), Negative control with water (Lanes 7 and 8), M0191 (Lanes 9 and 10), M0181 (Lanes 11 and 12), RDWG/MILAN (Lanes 13 and 14), Chinese Spring (Lanes 15 and 16), DL 2000 marker (lane 17) and Yunnong 202 (Lanes 18 and 19). *Pinb* fragment of 597 bp was expected in lanes 1, 3, 5, 7, 9, 11, 13, 15, 18 and *Pina* fragment with expected 524 bp was in lanes 2, 4, 6, 8, 10, 12, 14, 16, 19.

No PCR amplification with the 6th and 18th pairs of primers and the presence of PCR amplicons with the 5th and 19th pairs of primers indicated that the deletion occurred within the region between the 5th reverse primer and the 19th forward primer in wheat varieties Gaocheng 8901 and Shaan 253 (Fig. 1). A pair of primers spanning the deletion, namely *Pina-N*, was designed for detecting the *Pina-D1b* genotype. Fortunately, a 776-bp fragment was successfully amplified in Gaocheng 8901 and Shaan 253, respectively, while Chinese Spring and Yunong 202 which possess wild-type *Pina* did not produce any PCR fragment (Fig. 4). Furthermore, the 776-bp PCR fragment was also obtained in eight CIMMYT cultivars that were PINA null, which is in accordance with the results of SDS-PAGE and PCR amplification analysis with primers *Pina-D* and *Pinb-D*.

Sequencing plasmids containing the amplified fragments with marker *Pina-N* indicated that all 10 cultivars with the 776-bp PCR fragment possessed a single 15,380-bp deletion starting at $+23$ bp of the *Pina* gene, when compared with wild-type *Pina* and *Ha* sequence (soft wheat cv. Renan, NCBI CR262934) (Chantret et al., 2005).

Before this study, the PINA null phenotype was generally identified by SDS-PAGE, and varieties lacking PINA were assigned *Pina-D1b*. Although this result could be supported with lack of amplification using PCR primers for *Pina-D1a*, the results were still equivocal. Further, *Pina-D1b* is only one of a few possible PINA null-producing genotypes. Failure to generate a *Pina* amplicon may be a result of poor DNA quality or mistakes by operators etc. even if *Pinb-D1a* primers are used as a control for DNA quality. Therefore, the STS marker *Pina-N* developed in this study will be very useful to positively identify *Pina-D1b* alleles in bread wheat. Furthermore, it can also provide a helpful way, by using *Pina-N* for *Pina-D1b* along with *Pina-D* for *Pina-D1a*, to identify the homozygously, heterozygously positive and negative lines of breeding progenies of *Pina-D1a* and *Pina-D1b* alleles in the wheat breeding program.

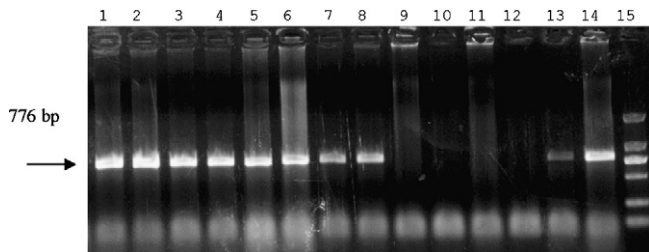


Fig. 4. Identification of *Pina-D1b* genotype with the STS marker Pina-N. From left to right: Shaan 253 (Lane 1), Gaocheng 8901 (Lane 2), Mo 299 (Lane 3), Mo293 (Lane 4), Mo215 (Lane 5), Mo191 (Lane 6), Mo181 (Lane 7), Mo272 (Lane 8), Chinese Spring (Lanes 9 and 11), Yunnong 202 (Lanes 10 and 12), RDWG/MILAN (Lane 13), CMH82A.1294/2*KAUZ//MUNIA/CHT O/3/MILAN (Lane 14), DL 2000 marker (Lane 15).

In this study, one of the important reasons that the primers were designed by primer walking strategy rather than directly from deletion fragment is that there might be more than one genotype with deletion fragment, resulting in a null of PINA protein. The speculation has been proven by the discovery of another allele with totally different deletion from *Pina-D1b* very recently (Chen, unpublished), even if uncovering the detailed deletion fragment is still in process.

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