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Allelic Status of *puroindoline*A and *puroindoline*B in Indian Bread Wheat Varieties

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ABSTRACT

'Soft' and 'hard' are classes of wheat (*Triticum aestivum*) and are distinguished by the texture of grain which in turn is due to expression of puroindoline genes *Pina* and *Pinb*, which are the main components of the 15 kDa friabilin protein that is associated with grain texture. Here we report the status of alleles of *Pina* and *Pinb* in 54 Indian and six Australian bread wheat varieties. The alleles were identified by PCR amplification of the *Pina* alleles and using PCR-CAPS markers for alleles *Pina-D1b* and *Pina-D1c* using restriction enzymes *Pvu*II and *BsrB*I respectively. *Pina-D1b* was found to be more frequent, *Pinb-D1b* was less frequent and *Pinb-D1c* was absent among the Indian varieties analyzed. None had mutations in both the puroindoline genes simultaneously. Analysis of a F_2 population segregating for the *Pina* (*Pina-D1a* and *Pina-D1b*) and *Pinb* (*Pinb-D1a* and *Pinb-D1b*) alleles showed the absence of recombination between the two loci, which is possibly due to tight linkage.

Keywords: hardness, PCR-CAPS, *Pina*, *Pinb*, *Triticum aestivum*
Abbreviations: CAPS, cleaved amplified polymorphic sequences; **PCR**, polymerase chain reaction

INTRODUCTION

Endosperm hardness is one of the most important quality characteristics of cultivated wheat (*Triticum aestivum*) and has a profound effect on milling, baking and quality of end product. Hard wheats are preferred for yeast-leavened bread making and soft wheats are preferred for cookies, cakes and pastries. The texture of the endosperm results mostly from the expression of a major locus designated 'Hardness' (Ha/ha) located on the short arm of chromosome 5D. At this locus several genes, notably the puroindolines, have been identified. The two major puroindoline genes *Pina* and *Pinb* are the major components of the 15 kDa protein friabilin (first reported by Greenwell and Schofield 1986), a starch granule protein associated with wheat endosperm softness (Giroux and Morris 1998; Morris 2002; Capparelli *et al.* 2003).

When wild type alleles for both the puroindolin genes are present the wheat endosperm is soft, where as it is very hard when both the genes carry mutation, which lead to changes in expression pattern and/or Pin protein structure. However, when one of the two genes is mutated, the endosperms show intermediate hardness. A number of alleles for *Pina* and *Pinb* have been identified in wheat (Wang *et al.* 2008; Morris and Bhave 2008; Ikeda *et al.* 2010). Wang *et al.* (2008) analyzed a micro core collection of China by an ecotilling approach and found three *Pina* alleles and eight *Pinb* alleles which included *Pinb-D1x* a novel variant. Morris and Bhave (2008) gave a detailed account of the *Pina* and *Pinb* alleles reported in the literature. They listed 18 variants of *Pinb-D1a* from *T. aestivum.* Among the several variants of *Pina-D1a* listed, seven were from *T. aestivum*. Among these variants from *T. aestivum*, *Pina-D1b*, a null allele, is due to a deletion of 15.38 kbp including *Pina* gene. They also listed a novel allele, *Pina-D1k*, which has a large deletion that includes *Pina*, *Gsp* and *Pinb*. Two more *Pina* null mutations involving 4.4 and 10.4 kbp deletions have been recently reported among Asian common wheat cultivars (Ikeda *et al.* 2010). Among the prominent *Pina* alleles is the *Pina-D1b* (null allele) which is due to a large deletion in the region, including *Pina*, and alleles of *Pinb-D1a* viz. *Pinb-D1b* due to a point mutation leading to gly-46 ser mutation (Giroux and Morris 1997), *Pinb-D1c* due to a point mutation leading to leu-60 pro mutation (Lillemo and Morris 2000) and *Pinb-D1d* due to a point mutation leading to trp-44 arg mutation (Lillemo and Morris 2000). A frame shift mutation caused by one base insertion and resulting in a change from glutamic acid to glycine at position 14 and stop codon at position 49 in *Pinb* in two Indian cultivars resulting in hardness has also been reported (Ram *et al.* 2005).

The status of *Pina* and *Pinb* alleles in many common wheat germplasm from different regions of the world such as China, Australia, Shandong, Asian etc have been reported

Table 1 List of wheat varieties used in this study.

Name of varieties	HDR-77, Unnath Kalyan Sona, HD-2428, Unnath Sonalika, HD-2668, HD-1949, HD-2643, UP-2338, HD-2385, HD-2285, HD-2745, HD-2320, GW-190, LOK 1, HD-1941, HD-1925, HD-2177, HD-2189, HD-2270, HD-2135, HD-2327, HD-2667, Vaishali, Vidhisha, C-306, Kundan, HPW-42, Agra local, Sehore, Ajanta, PBNS-3963, Parbhani-51, WH-542, PBW-175, Raj-3765, PBW-154, PBW-299, PBW-138, PBW-373, Kalyan Sona, Sonalika, HS-240, CPAN-1922, MACS-2496, PBW-343, Kanchan, HUW-206, Takari, Flinder, Kite, Harrier, Jabiru, King, Chinese Spring,
Source	NA&BT Division, Bhabha Atomic Research Center
Name of varieties	UP-262, HD-2009, WH-147, Lerma Rojo 64, WL-711, HD-1982
Source	National Bureau for Plant Genetic Resources (NBPGR), New Delhi

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Original Research Paper

(Pickering and Bhave 2007; Li *et al.* 2008; Wang *et al.* 2008; Ikeda *et al.* 2010). There are few reports of the status of *Pina* and *Pinb* alleles among Indian wheat germplasm (Ram *et al.* 2002, 2005). It has been observed that *Pina-D1b* (null allele) is a predominant genotype in CIMMYT wheats and is popular in Chinese, Latin American and Indian wheat germplasm (Ram *et al.* 2002; Li *et al.* 2008). Specific primers are available to PCR-amplify *Pina* and *Pinb* genes and to also trace the specific allele for the mutations in *Pinb*. Using these primers Ram *et al.* (2002) analyzed the *Pina* and *Pinb* genes in 100 Indian bread wheat varieties and reported that null mutation in *Pina* is prevalent in Indian wheats. However, the discrimination of the primers to trace the alleles in *Pinb* is based on a single base pair difference at the 3′ end of the primers and such allele specific amplifications occasionally gives false positives unless stringent PCR conditions are used. To avoid this problem co-dominant PCR-CAPS marker based on the same point mutation has been developed (http://maswheat.ucdavis.edu), which depends on the PCR amplification of the gene using the universal primer and analyzing the product by digesting with a restriction site. In this study we report the status of the *Pina* and *Pinb* alleles in some recently released Indian bread wheat varieties using the PCR-CAPS markers. The *Pina* and *Pinb* allelic status of the Chinese Spring identified by this method is in agreement with their known allelic status.

MATERIALS AND METHODS

Plant materials

Bread wheat (*Triticum aestivum* L.) varieties used in this study are listed in **Table 1**. The varieties included 54 Indian (serial number 1-54 in **Table 2**), six Australian (serial number 55-60 in **Table 2**) and Chinese Spring. The mapping population consisting of 150 F_2 plants was from a cross between Sonalika and Kalyan Sona (Nalini *et al*. 2007). All the wheat varieties and the F_2 population were grown at Trombay under field conditions.

Chemicals

Agarose, Tris, EDTA, sodium chloride, urea, SDS, isopropanol, sodium acetate, chloroform, isoamylalcohol and phenol were obtained from Sigma-Aldrich Corporation, St. Louis, Missouri USA. *Taq* DNA Polymerase and dNTPs were from Bangalore Genei Ltd. Bangalore, India. Hoechst dye was from Amersham Pharmacia Biotech, India. The restriction enzymes *Pvu*II and *Bsrb*I were from New England Biolabs.

DNA extraction and estimation

DNA was isolated from the young leaves according to the method of Nalini *et al.* (2004). The method essentially involves extraction using a buffer containing urea, SDS, Tris, EDTA, sodium chloride and β-mercaptoethanol. This was followed by purification with phenol: chloroform mixture. DNA was precipitated using sodium acetate and isopropanol. The DNA pellet was rinsed with 70% followed by 100% ethanol, dried in air and dissolved in Tris-EDTA buffer. After RNAase treatment to degrade RNA, the DNA was estimated by measuring the fluorescence emission using the dye Hoechst. The fluorescence intensity was measured at emission maximum of 458 nm using a fluorimeter (Hoefer DyNA Quant 200).

PCR of *Pina* and *Pinb* genes

The *Pina* and *Pinb* were PCR amplified using primers designed by Gautier *et al.* (1994; MASwheat 2009) viz., *Pina*-D1-F: 5'- CCC TGT AGA GAC AAA GCT AA -3' and *Pina*-D1-R: 5'- TCA CCA GTA ATA GCC AAT AGT G -3', for *Pina* and *Pinb*-D1-F: 5'- ATG AAG ACC TTA TTC CTC CTA -3' *Pinb*-D1-R: 5'- TCA CCA GTA ATA GCC ACT AGG GAA -3' for *Pinb*. The reaction was performed in 25 µl containing 10 pmoles of each primer, 200 µm of dNTPs, 2 mM of $MgCl_2$, 1 unit of *Taq* DNA polymerase and 100 ng of template DNA. The PCR amplification was carried out in an Eppendorf Mastercycler according to the cycling conditions described by MASwheat (2009). The PCR products were separated on a 2% agarose gel prepared in 1X TBE buffer, visualized under UV light after ethidium bromide staining and photographed (Singh and Jawali 2009). The expected sizes of the PCR products for *Pina* and *Pinb* were 330 and 447 bp, respectively.

Table 2 Allelic status of *Pina* and *Pinb* in Indian and Australian bread wheat varieties.

Name of variety	Year of release	PIN A (330 bp)	Alleles of *PINA*	Alleles of *PINB*
WH-542	1995	0	*Pina*-D1b	*Pinb*-D1a
HDR-77	1990	0	*Pina*-D1b	*Pinb*-D1a
Unnath Kalyan Sona	-	0	*Pina*-D1b	*Pinb*-D1a
HD-2428	1988	0	*Pina*-D1b	*Pinb*-D1a
PBW-175	1988	1	*Pina*-D1a	*Pinb*-D1*
Unnath Sonalika	-	1	*Pina*-D1a	*Pinb*-D1b
HD-2668	-	0	*Pina*-D1b	*Pinb*-D1a
HD-1949	1974	1	*Pina*-D1a	*Pinb*-D1b
HD-2643	1995	0	*Pina*-D1b	*Pinb*-D1a
Raj-3765	1994	0	*Pina*-D1b	*Pinb*-D1a
PBW-154	1986	0	*Pina*-D1b	*Pinb*-D1a
UP-2338	1995	1	*Pina*-D1a	*Pinb*-D1*
HD-2285	1991	1	*Pina*-D1a	*Pinb*-D1*
HD-2745	-	0	*Pina*-D1b	*Pinb*-D1a
Ajanta	-	0	*Pina*-D1b	*Pinb*-D1a
HD-2735	-	1	*Pina*-D1a	*Pinb*-D1*
C-306	1965	0	*Pina*-D1b	*Pinb*-D1a
PBNS-3963	-	0	*Pina*-D1b	*Pinb*-D1a
HD-2320	1985	0	*Pina*-D1b	*Pinb*-D1a
GW-190	1994	0	*Pina*-D1b	*Pinb*-D1a
LOK 1	-	1	*Pina*-D1a	*Pinb*-D1b
HUW-206	1985	0	*Pina*-D1b	*Pinb*-D1a
PBW-299	1992	0	*Pina*-D1b	*Pinb*-D1a
Parbhani-51	-	0	*Pina*-D1b	*Pinb*-D1a
HD-1941	1970	0	*Pina*-D1b	*Pinb*-D1a
PBW-138	1986	0	*Pina*-D1b	*Pinb*-D1a
Kanchan	1994	0	*Pina*-D1b	*Pinb*-D1a
HD-1925	1974	0	*Pina*-D1b	*Pinb*-D1a
HD-2177	1978	0	*Pina*-D1b	*Pinb*-D1a
HD-2189	1979	0	*Pina*-D1b	*Pinb*-D1a
HD-2270	1987	0	*Pina*-D1b	*Pinb*-D1a
PBW-373	1995	0	*Pina*-D1b	*Pinb*-D1a
HD-2135	1975	1	*Pina*-D1a	*Pinb*-D1*
HD-2327	1985	0	*Pina*-D1b	*Pinb*-D1a
HD-2667	-	0	*Pina*-D1b	*Pinb*-D1a
HD-2172	1981	0	*Pina*-D1b	*Pinb*-D1a
Sehore	-	0	*Pina*-D1b	*Pinb*-D1a
HD-1982	1974	0	*Pina*-D1b	*Pinb*-D1a
UP-262	1977	0	*Pina*-D1b	*Pinb*-D1a
WL-711	1979	0	*Pina*-D1b	*Pinb*-D1a
HD-2009	1975	0	*Pina*-D1b	*Pinb*-D1a
Lerma Rojo 64	1965	1	*Pina*-D1a	*Pinb*-D1*
WH-147	1977	0	*Pina*-D1b	*Pinb*-D1a
PBW-343	1994	0	*Pina*-D1b	*Pinb*-D1a
MACS-2496	1991	0	*Pina*-D1b	*Pinb*-D1a
HPW-42	1993	1	*Pina*-D1a	*Pinb*-D1*
CPAN-1922	1984	0	*Pina*-D1b	*Pinb*-D1a
Kundan	1985	0	*Pina*-D1b	*Pinb*-D1a
HS-240	1988	0	*Pina*-D1b	*Pinb*-D1a
Vidhisha	1997	0	*Pina*-D1b	*Pinb*-D1a
Kalyan Sona	1967	0	*Pina*-D1b	*Pinb*-D1a
Agralocal	-	1	*Pina*-D1a	*Pinb*-D1a
Vaishali	1993	0	*Pina*-D1b	*Pinb*-D1a
Sonalika	1967	1	*Pina*-D1a	*Pinb*-D1b
Takari	-	0	*Pina*-D1b	*Pinb*-D1a
Flinder	-	1	*Pina*-D1a	*Pinb*-D1b
Kite	-	1	*Pina*-D1a	*Pinb*-D1b
Harrier	-	1	*Pina*-D1a	*Pinb*-D1b
Jabiru	-	1	*Pina*-D1a	*Pinb*-D1*
King	-	1	*Pina*-D1a	*Pinb*-D1b
Chinese Spring	-	1	*Pina*-D1a	*Pinb*-D1a

Note: *Pinb**= the allele is neither *Pinb-D1b* or *Pinb-D1c*

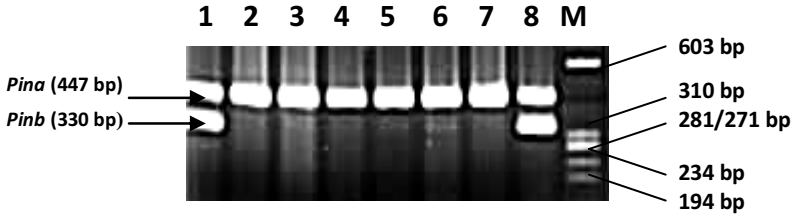

Fig. 1 PCR amplified products of *Pina* and *Pinb* obtained among eight wheat varieties. Lane 1: Lok 1; 2: HUW-206; 3: PBW-299; 4: Parbhani-51; 5: HD-1941; 6: PBW-138; 7: Kanchan; 8: Chinese Spring. The PCR amplification of *Pina* and *Pinb* was carried out as described in Materials and Methods. Lane M; *Hae*III digested φX174 DNA.

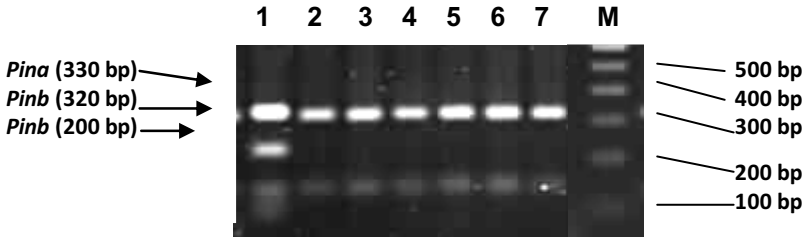

Fig. 2 *Bsrb*I-digested fragments of PCR amplified products of *Pinb* obtained from eight wheat varieties. Lane 1: Lok 1; 2: HUW-206; 3: PBW-299; 4: Parbhani-51; 5: HD-1941; 6: PBW-138; 7: Kanchan. The PCR amplification products of *Pina* and *Pinb* were digested with *Bsrb*I as described in Materials and Methods. Lane M: 100-bp ladder.

Restriction enzyme cleavage of PCR-amplified Pinb-D1 genes for identification of alleles

Pinb alleles were identified through analysis of two different cleaved amplified polymorphic sequences (CAPS) sites and using the established diagnostic restriction fragment length polymorphisms reported in the literature (Lillemo and Morris 2000; MASwheat 2009). *Pinb-D1b* was identified by digesting the PCR product of *Pinb* with restriction enzyme *Bsrb*I (MASwheat 2009), and *Pinb-D1c by* digesting the PCR product with restriction enzyme *Pvu*II (Lillemo and Morris 2000). An aliquot of the PCR product equivalent to ~ 1000 ng was digested in a final reaction volume of 10 µl with 2U of the enzyme and the buffer supplied by the manufacturer. After overnight incubation at 37°C the digested products were separated on a 2% agarose gel prepared in 1X TBE buffer, visualized under UV light after ethidium bromide staining and photographed (Singh and Jawali 2009).

RESULTS

The PCR amplification of *Pina* and *Pinb* individually using Chinese Spring DNA as template yielded two fragments of expected size 330 and 447 bp, respectively. As the conditions for PCR were same for both *Pina* and *Pinb*, multiplex-PCR was carried out in which the primers for both *Pina* and *Pinb* were added together and the reaction was performed using thermal cycling conditions as given in Materials and Methods. As an example, amplified products of *Pina* and *Pinb* from the multiplex PCR of eight wheat varieties is shown in **Fig. 1**. As expected Chinese Spring yielded two DNA fragments of size 330 and 447 bp corresponding to *Pina* and *Pinb*, respectively. Results showed that 41 out of the 54 Indian wheat varieties carried *Pina-D1b* null allele. Similar findings were also reported by Ram *et al.* (2002). In contrast, five out of the six Australian cultivars analyzed had the wild type *Pina* viz. *Pina-D1a*. In contrast to this, the DNA fragment of 447 bp in size from *Pinb* gene was found in all the 61wheat varieties showing the absence of *Pina-D1k* (Tranquilli *et al*. 2003; Chang *et al*. 2006; Morris and Bhave 2008) or a null allele of *Pinb* in the varieties analyzed. Two mutations in *Pinb*, represented by *Pinb-D1b* and *Pinb-D1c*, lead to a change in amino acid gly-ser at position 46 and leu-pro at position 60 respectively, confer hardness to the grains (Lillemo and Morris 2000; MASwheat 2009). Analyses showed that the *Pvu*II and *Bsrb*I sites were absent in *Pina-D1*, which allowed us to use the multiplex-PCR amplification product for analyzing the *Pinb-D1b* and *Pinb-D1c* alleles. This was further confirmed by digesting *Pina-D1a* PCR product from six wheat varieties. The wheat varieties were screened for the two *Pinb* alleles by using the multiplex-PCR product as described under Materials and Methods.

We subjected the *Pina* and *Pinb* multiplex PCR product of all the varieties to restriction digestion with enzyme *Bsrb*I for identifying *Pinb-D1b*. After digestion, fragments of 320 bp are expected for varieties lacking the glycine to serine mutation, while fragments of 200 bp are expected for those varieties carrying the allele (*Pinb-D1b*) for hard texture of the grain (MASwheat 2009). The *Bsrb*I profile obtained for a few varieties is shown in **Fig. 2**. LOK-1 variety in lane 1 showed the presence of the 200-bp band along with multiple small bands indicated the presence of *Pinb-D1b* allele. All the other varieties from lane 2 to 7 showed the 320-bp band along with a smaller 130-bp band, which indicated that they did not carry the mutation and hence did not carry *Pinb-D1b* allele. The majority of Indian wheat varieties did not carry the *Pinb-D1b* allele except for Unnath Sonalika, HD-1949, LOK-1 and Sonalika. Among the Australian varieties four were found to carry the *Pinb-D1b* allele.

In the *Pinb-D1a* sequence there is one *Pvu*II site corresponding to the 60th amino acid proline and hence the PCR amplified *Pinb-D1a* from the wild type would yield two fragments (264 and 184 bp). However, the PCR product from genotypes supposedly carrying *Pinb-D1c* would have a point mutation within the *PvuII* recognition sequence (leading to a change in leu-60 proline) and hence would fail to be digested by *Pvu*II. Therefore, failure to cut at the restriction site indicated the presence of this mutation and hence identified as *Pinb-D1c* (Lillemo and Morris 2000). Upon digestion with *Pvu*II the multiplex PCR product, only the *Pinb* product of all the varieties yielded two fragments (264 and 184 bp), indicating that none of the varieties analysed carried *Pinb-D1c*. As an example, the DNA fragment profile of the PCR product of a few wheat varieties digested with *Pvu*II is shown in **Fig. 3**.

Allelic information for *Pina* and *Pinb* genes obtained for the varieties analyzed is given in **Table 2**. Among the Indian varieties studied, *Pinb-D1c* allele was absent where as *Pinb-D1b* was rare. Among the Australian varieties analyzed the *Pinb-D1c* allele was absent but *Pinb-D1b* was prevalent.

To date in all the varieties analyzed from different countries, it has been observed that the occurrence of mutations

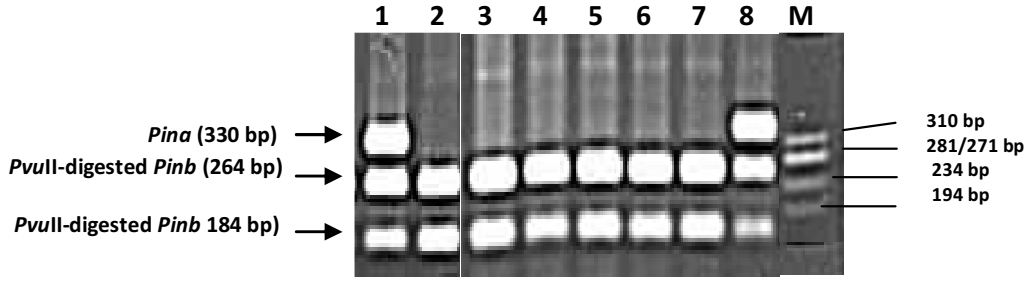

Fig. 3 *Pvu*II-digested fragments of PCR amplified products of *Pinb* obtained from eight wheat varieties. Lane 1: Lok 1; 2: HUW-206; 3: PBW-299; 4: Parbhani-51; 5: HD-1941; 6: PBW-138; 7: Kanchan; 8: Chinese Spring. The PCR amplification products of *Pina* and *Pinb* were digested with *Pvu*II as described in Materials and Methods. Lane M; *Hae*III digested φX174 DNA.

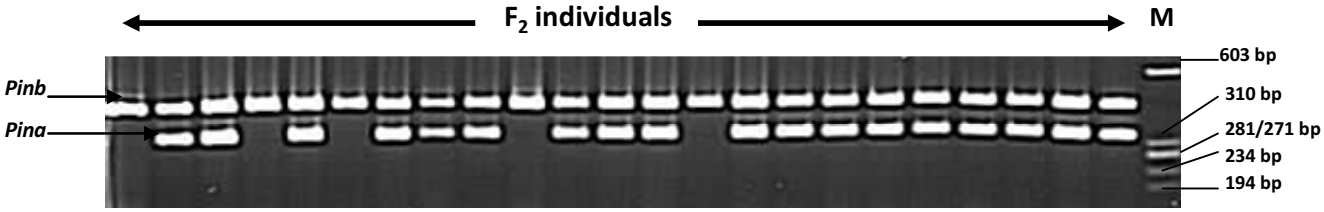

Fig. 4 Segregation of *Pina-D1a* allele and the null allele *Pina-D1b*. The segregation of the *Pina* and *Pinb* alleles among 23 individuals from a cross between Sonalika and Kalyan Sona Lane M; *Hae*III-digested φX174 DNA.

in *Pina* and *Pinb* are mutually exclusive (Lillemo and Morris 2000). There are no reports on the recombination between *Pina* and *Pinb* mutants studied by analyzing a segregating population. The varieties Kalyan Sona and Sonalika harbor *Pina-D1b/Pinb-D1a* and *Pina-D1a/Pinb-D1b*, respectively. We analyzed a population of 150 F_2 individuals derived from a cross between Sonalika and Kalyan Sona for the *Pina* and *Pinb* recombinants. PCR analysis revealed that *Pina-D1a* was present in 115 F_2 individuals and the remaining 35 F_2 individuals showed the null allele *Pina-D1b* only, which showed an expected segregation of 3: 1. As an example the segregation of the *Pina* alleles in 23 F_2 individuals is shown in **Fig 4**.

The *Pinb* product of 447-bp size was present in all F_2 individuals, as both the parents yield a *Pinb* product. As the individuals showing *Pina* null allele (*Pina-D1b*) are homozygous for *Pina*, whether the 35 F_2 individuals carrying null *Pina* allele carry *Pinb-D1b* was analyzed by digesting the multiplex PCR product with the restriction enzyme *Bsrb*I. None of the 35 F_2 individuals had *Pinb-D1b* as judged from the DNA profile indicating the absence of recombination between *Pina* and *Pinb* in the population analyzed.

DISCUSSION

Wheat (*Triticum aestivum*) grain texture influences the technological characteristics of wheat grain, particularly milling properties. Soft-textured wheat typically has increased break flour yield, a smaller flour particle size and less starch damage compared to hard-textured wheat. Therefore flour from hard wheat is preferred for making bread, whereas flour from soft wheat is more suitable for producing biscuits, cookies and cakes (Ma *et al.* 2009). Both *Pina* and *Pinb* content have been shown to have a significant influence on dough properties (Dubreil *et al*. 1998). Studies have shown that puroindolines play a significant role in bread making i.e. they are involved in dough strength, bread notation and loaf volume (Igrejas *et al*. 2001, 2002). *Pinb* has been a major quantitative trait locus for milling and cookie baking traits (Ma *et al*. 2009). QTLs have been mapped not only on chromosome 5D but also on chromosome 7A (Wilkinson *et al.* 2008). Hence knowing the status of *Pina* and *Pinb* alleles in Indian cultivated varieties will be useful in breeding as well as to study the 'Hardness' locus.

In this paper we report the use of multiplex PCR for *Pina* and *Pinb* alleles analyses in wheat. The success of the multiplex PCR analyses is due to a) the primers for both *Pina* and *Pinb* have same PCR amplification condition b) the *Pina* and *Pinb* product size being distinctly different and c) presence of *Pvu*II and *Bsr*I sites only in *Pinb* and not in *Pina.* Multiplexing saves both the PCR amplification and the agarose gel electrophoresis and hence significantly decreases time taken for analyses. Multiplex-PCR is also particularly important as *Pinb* amplification serves as an internal control for PCR amplification to rule out that non-amplification of *Pina* as in the case of *Pina-D1b* allele is due to PCR failure. Chang *et al*. (2006) also used multiplex-PCR to analyze *Pina* and *Pinb* alleles; however, it did not involve the use of CAPS. The allelic status for some Indian and Australian varieties identified in this study was found to be same as that was reported by others (Lillemo *et al*. 2006; Pickering and Bhave 2007). Pickering and Bhave (2007) reported the allele status for Harrier, Kite and Kalyan Sona and Lillemo *et al*. (2006) the status of Kalyan Sona, Sonalika, PBW 343, Lok 1 and Kanchan.

The analysis of the Indian wheat varieties showed that majority of them harbored *Pina-D1b* and it was also observed that the varieties had either *Pina-D1b* or *Pinb-D1b* as a gene for hardness but never both together. We are for the first time reporting the analyses of a segregating F_2 population derived from the cross Sonalika (*Pina-D1a/ Pinb-D1b*) and Kalyan Sona (*Pina-D1b/Pinb-D1a*) to study recombination involving *Pina* and *Pinb*. Absence of a recombinant in the population analyzed could be due to a) mutation in both *Pina* and *Pinb* is lethal and b) that the two are closely linked. The former reason is ruled out as Tranquilli *et al.* (2002) obtained a line with simultaneous deletions in both the puroindoline loci and showed that average hardness indexes ~5% higher than those observed in genotypes with individual mutations. Chang *et al.* (2006) subsequently reported six "double nulls" in some wheat cultivars, which is due to a large deletion encompassing both *Pina* and *Pinb*. The absence of PCR amplification in both *Pina* and *Pinb* genes has now been designated as *Pina-D1k* (Morris and Bhave 2008). The later one is likely to be the reason as the distance between *Pina* and *Pinb* is less than 20 kbp (Ikeda *et al*. 2010) and hence one may need to screen a larger F_2 population. Our results showed that *Pina-D1k* was not found among the Indian wheat varieties analysed and indicated that it may not be common among the Indian wheats.

The *Pina* wild type allele (*Pina-D1a*) was predominant

in the Australian cultivars analyzed in this study. Even though the number of varieties analysed was small the result obtained is in agreement with the study conducted by Pickering and Bhave (2007) where they analyzed 55 Australian cultivars and found 33 (60%) of them to have the *Pina-D1a* allele indicating that mutation in *Pinb* is more prevalent than in *Pina*. Seven Indian varieties, viz. PBW-175, UP-2338, HD-2285, HD-2735, HD-2135, Lerma Rojo 64, HP-42 and Jabiru showed exceptions by possessing the wild type *Pina* allele (*Pina-D1a*) and the glycine type *Pinb* (soft type). In these varieties there may be additional mutations, which have to be analyzed depending on which the allelic status of *Pinb* may change.

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