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ORIGINAL ARTICLE

Marker-assisted transfer of *PinaD1a* gene to develop soft grain wheat cultivars

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Abstract

Grain softness has been a major trait of interest in wheat because of its role in producing flour suitable for making high-quality biscuits, cookies, cakes and some other products. In the present study, marker-assisted backcross breeding scheme was deployed to develop advanced wheat lines with soft grains. The Australian soft-grained variety Barham was used as the donor parent to transfer the puroindoline grain softness gene *Pina-D1a* to the Indian variety, DBW14, which is hard grained and has *PinaD1bPinbD1a* genes. Foreground selection with allele-specific PCR-based primer for *Pina-D1a* (positive selection) was used to identify heterozygous BC_1F_1 plants. Background selection with 173 polymorphic SSR primers covering all the 21 chromosomes was also carried out, in the foreground-selected BC_1F_1 plants. BC_1F_2 plants were selected by ascertaining the presence of *Pina-D1a* (positive selection) and absence of *Pina-D1b* (negative selection). Using the approach of positive, negative and background selection with molecular markers, 15 BC_1F_2 and 31 BC_2F_1 plants were finally selected. The 15 BC_1F_2 plants were selfed and the 31 BC_2F_1 plants were further backcrossed and selfed to raise BC_3F_1 and BC_2F_2 progenies, respectively. A part of the BC_2F_2 seed of each of the 31 plants was analyzed for grain hardness index (GHI) with single-kernel characterization system. The GHI varied from 12.1 to 37.1 in the seeds borne on the 31 BC_2F_1 plants. The reasons for this variation and further course of action are discussed.

Keywords Grain softness · Marker-assisted backcross breeding · Puroindolines · Wheat grain quality

Introduction

Wheat is an important source of major nutrients required by the human body. The wheat kernel contains roughly 12% water, 70% carbohydrates, 12% protein, 2% fat, 1.8% minerals and 2.2% crude fibers (The Editors of Encyclopaedia Britannica 2018). Wheat is the second important food crop next to rice in India and contributes to nearly 25% of food grain production of the country (FAO in India 2018). Nearly 80% of wheat is consumed by Indian people in the form of 'chapatti': an unleavened flat bread (Misra 1998). Indian wheat is not segregated according to the end-product requirement though some amount of segregation does take place on the basis of grain color, hardness and luster which is sold at a higher price. The wheat breeding programs in India have been mainly focused on yield and disease resistance to develop verities to feed the ever-growing population. In the recent years, however, the milling and bakery industry in India has grown enormously (Indian Bakery Market 2018) and instant and ready-to-eat wheat-based foods have become popular. Several shopping malls, airports and similar centers have come up which has led to the establishment of bakeries serving ready-to-eat food. There is an increased production of breads, biscuits, cakes, buns, noodles, etc., in the country. India is now the world's third largest country producing biscuits after USA and China (Kachave 2018). However, getting the desired quality of wheat flour for making specific

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kinds of bakery products is a challenging task for bakery industries. This results in use of external agents including potentially harmful chemicals for improving the texture and quality of end-products. The inconsistency in different wheat or flour lots also poses a challenge to get a consistent quality for baking. The need for development of product-specific varieties in the country has therefore been felt. The dearth of breeders, in the country, engaged in wheat quality enhancement makes this task difficult to achieve.

The transfer of quality traits during the development of elite genotypes or to the already released cultivars is time-consuming and inefficient by the conventional strategy (Hospital 2005). Moreover, most quality tests can be conducted only in the advanced generations, thereby increasing the time required to select the appropriate lines. Rapid advances in genomic research and molecular biology have led to the development of precise, rapid and efficient molecular markers for speedy development of new cultivars (Randhawa et al. 2009). Linked and gene-based markers have also become available for several quality traits in wheat (Ma et al. 2003; Wang et al. 2009, 2010). Therefore, combining marker-assisted selection with conventional phenotypic selection can enhance the efficiency of breeding and precise transfer of the target allele into the advanced progenies and sometimes in relatively shorter time (Hospital and Charcosset 1997).

A flour derived from soft grain endosperm with < 11% flour protein and producing weak and extensible gluten is considered ideal for biscuit quality and the alveo and farinographic indices, and the alleles of high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) have been suggested to achieve these criteria (Payne 1987; Rasheed et al. 2014). In our earlier study (Rai et al. 2018 communicated), 30 wheat varieties were screened for quality parameters and allele-specific PCR markers of *PinaD1a*, *PinaD1b*, *PinbD1a*, *PinbD1b* for grain texture and *Glu-1* and *Glu-3* genes for gluten strength and extensibility to identify candidate Indian wheat varieties for improving grain endosperm texture. Varieties HS490, HI1563 and DBW14 were found to possess desirable values of some of the related traits such as HMW-GS, LMW-GS, SDS-sedimentation value and farinograph quality number (FQN), and their values approached those in the Australian soft endosperm wheat lines Barham and Longreach Orion. Additionally, the variety DBW14 possessed HMW-GS (*GluA1a, GluB1u, GluD1d*) and LMW-GS (*GluA3c, GluB3b, GluD3c*) which were same as those of the Australian soft endosperm wheat varieties Barham and Longreach Orion and was therefore selected as the candidate variety to impart to it grain softness trait as it is a hard-grained variety. In this paper, we describe the process of marker-assisted backcross breeding (MABB) for grain softness trait with foreground and background markers in a DBW14/Barham cross.

Materials and methods

Plant materials

DBW14, an Indian awned wheat variety, was used as a recurrent parent (referred to as RP), and an Australian awnless variety, Barham, obtained in a collaborative program on marker-assisted breeding in wheat (Project no. 100157) was used as a donor parent (referred to as DP). DBW14 (RAJ 3765/PBW 343) is a semi-dwarf wheat variety released in the year 2002 for late sown irrigated conditions of North Eastern Plains Zone of India. These parents have been characterized in an earlier study by us (Rai et al. 2018, communicated). DBW 14 recorded a protein content of 10.19% in the crop season 2014–2015. It is a hard-grained variety and recorded a hardness index (HI) of 72.29 on single-kernel characterization system and has the allelic composition *PinaD1b* and *PinbD1a*. The donor parent Barham (Syn VO2697R) with the pedigree Bowie//Bersee/3*Bindawarrala 12697///Bowie is a wheat variety having moderate resistance to leaf and stem rust and possesses alien chromosome segment having *Lr37/Yr17/SR38* genes. It is soft grained with HI of 26 on single-kernel characterization system (SKCS) and possesses the wild-type alleles (*a*) of the genes *PinaD1* and *PinbD1*. Both DBW14 and Barham have similar alleles of HMW-GS and LMW-GS coding genes, and therefore no change in HMW or LMW alleles of DBW 14 would occur.

Grain characteristics

The parameters grain hardness index (GHI), thousand kernel weight (TKW) and diameter (DIA) were analyzed with single-kernel characterization system (SKCS) 4100 (Perten Instruments, Australia) using the procedure described in AACC (2000) method 55-31. Grain hardness was determined in each genotype on an approximately 300-kernel sample size. To determine TKW, 250 grains were taken at random, representing the whole sample, counted and weighed. The value so obtained was multiplied by four to get the 1000 kernel weight in 'grams.'

Genotyping

Markers for the alleles of the grain texture genes *Pina-D1a* (Gautier et al. 1994), *Pina-D1b* (or null) (Feng et al. 2013) and *PinbD1a* (Giroux and Morris 1997) were validated in our earlier work (Rai et al. 2015). Two of the markers were

used for the foreground selection (FG). The marker PinA (Gautier et al. 1994) was used for identifying the presence of grain softness gene *Pina-D1a*. Additionally, negative selection with a marker Pina-N1 (Feng et al. 2013) for *Pina-D1b* was carried out only in BC_1F_2 to identify homozygous individuals for *PinaD1a.* Primers were got synthesized by Sigma Pvt Ltd, India. PinA primer amplifies a ~ 450 bp fragment indicating the presence of *Pina-D1a* allele while Pina-N1 primer amplifies a 776 bp fragment indicating the presence of *Pina-D1b* allele. Supplementary Table 1 lists the primers, their respective sequence and fragment size obtained. A total of 1110 simple sequence repeat (SSR) markers covering all the 21 linkage groups of wheat were screened in the parents to find polymorphic SSR markers for use in background selection (BG) for the recovery of recurrent parent genome (RPG) in the segregating backcross progenies. The SSR primer sequences were obtained from http://wheat.pw.usda.gov/GG2/index.shtml (Grain Genes 2.0: a database for *Triticeae* and *Avena*) (Röder et al. 1998; Somers et al. 2004).

Marker-assisted backcross breeding

DNA isolation, PCR conditions and electrophoresis

DNA extraction of parental genotypes and their backcross or selfed progenies planted in the field was carried out from the leaves of 23–27-day-old plants using cetyltrimethylammonium bromide (CTAB) procedure (Doyle and Doyle 1987). PCR for PinA and Pina-N1 was performed using 0.3 µl Taq DNA polymerase in 15 µl reaction volumes containing approximately 50 ng of genomic DNA, 1 × PCR buffer with 1.5 $MgCl_2$, 10 pmol of each PCR primer and 200 µM of each of dNTPs. PCR cycling conditions were 94 °C for 5 min following 35 cycles of 94 °C for 35 s, 60–65 °C for 35 s, 72 °C for 90 s and a final extension at 72 °C for 8 min. The PCR products were separated by electrophoresis in 1.5% agarose for foreground selection. For the background selection, the PCR products were run on 3% metaphor and visualized by ethidium bromide staining.

Breeding scheme

MABB scheme was followed to transfer *Pina-D1a* gene from Barham into the genetic background of DBW14 and is depicted in Fig. 1. Recurrent parent (RP) DBW14 with *PinaD1b* allele was used for crossing with Barham as donor of *Pina-D1a* allele. Although DBW14 was used as a female in every backcrosses made so as to get the cytoplasmic content of the recurrent parent to attain maximum genome recovery, in producing F_1's, DBW14 was used

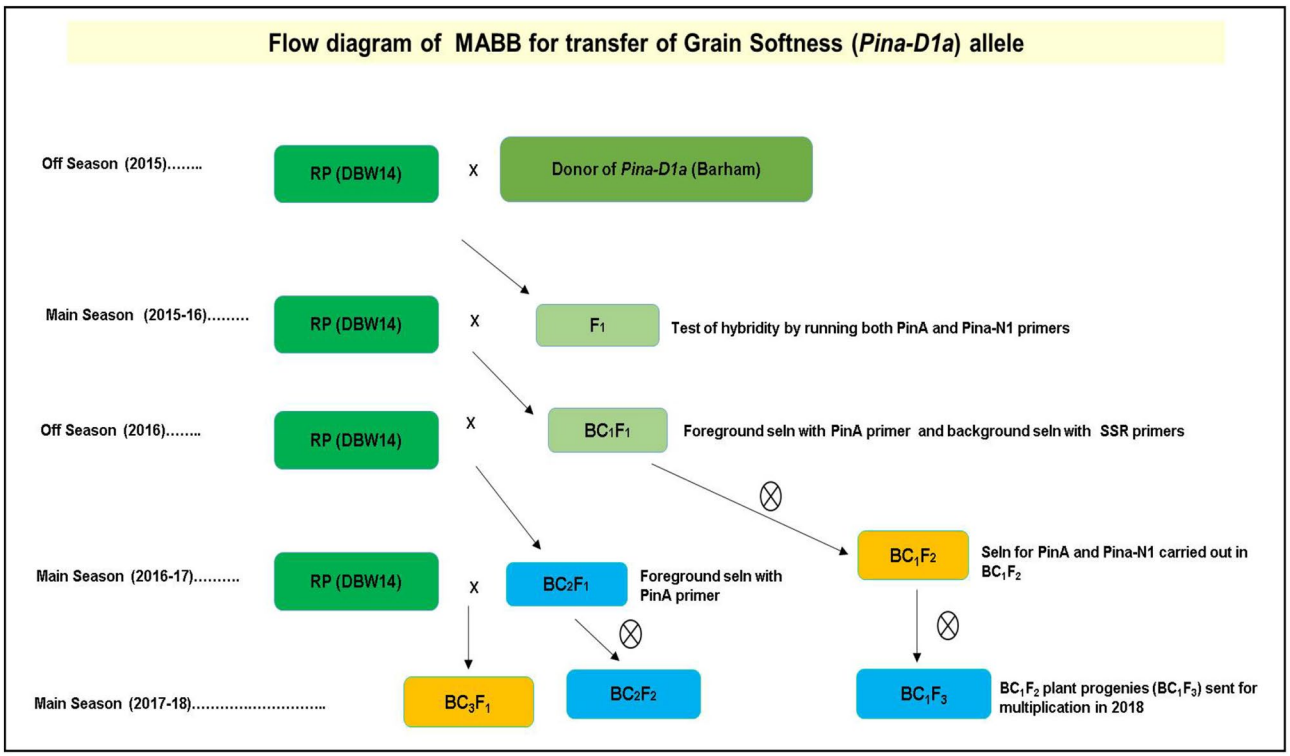

Fig. 1 Flowchart of marker-assisted backcross breeding for transfer of *Pina-D1a* allele

as pollen parent and Barham as female. Both foreground and background selections were carried out with rigorous molecular markers-based selection. The BC_1F_3 and BC_2F_2 seeds were generated till the submission of this article.

Determination of recurrent parent genome recovery

The extent of RPG recovery in backcross generation was calculated using the following formula:

$$\text{RPG}\% = \frac{2(R) + (H)}{2N} \times 100,$$

where R is the number of marker loci homozygous for recurrent parent allele; H is the number of marker loci remaining heterozygous and N is the total number of polymorphic markers used for background analysis (Miah et al. 2014). To represent RPG in the chromosome of the backcross progenies, Graphical Geno Type (GGT) software version 2 (Berloo 1999) was used.

Results

Marker-assisted backcross breeding for *PinaD1a* (foreground selection)

The cross was made between DBW14 which was used as pollen parent and Barham as female. Barham was used as female parent so that the selfed (*PinaD1aPinaD1a*) and the crossed seeds (*PinaD1aPinaD1b*) could be distinguished both morphologically and with markers for *PinaD1a* and *PinaD1b*. PINA primer was run in 105 F_1 plants, of which 99 plants showed (*Pina-D1a/Pina-D1b*) allele while six plants showed (*PinaD1a/PinaD1a*) allele. These six plants were homozygous for PINA locus showing that these were selfed seeds of Barham and were soft in appearance and therefore were discarded and not taken forward. The F_1 plants of the cross DBW14/Barham were screened with PinA (Fig. 2a). Further, DBW14 was used as a female in every backcrosses made so as to get the cytoplasmic content of the recurrent parent to attain maximum genome recovery. The 99 plants found positive for the allele (*Pina-D1a*) were backcrossed with RP, DBW14 to produce ~ 2000 BC_1F_1 seeds (Table 1). Out of these, 400 BC_1F_1 seeds were screened for foreground selection with PinA (Fig. 2b). Two hundred fifteen plants were positive for PinA. The 215 BC_1F_1 plants were strictly screened morphologically, and 112 plants were found to show

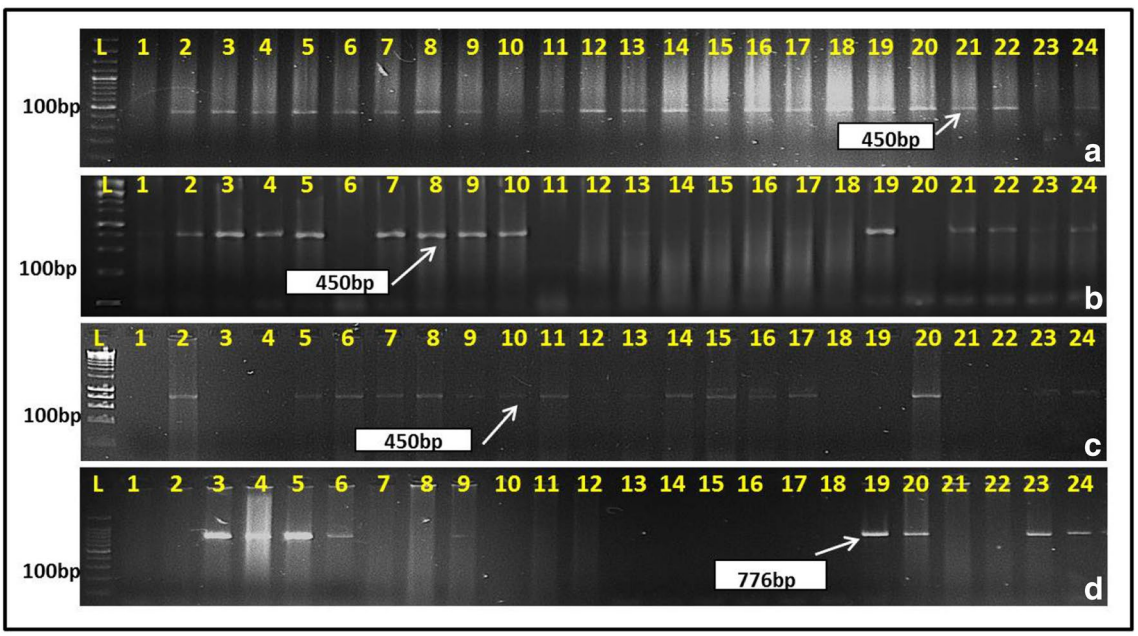

Fig. 2 PCR analysis for ascertaining the presence of target allele(s) (cropped gels displayed). **a** DNA amplification of *Pina-D1a* allele using the primer PinA in F_1 population, **b** DNA amplification of *Pina-D1a* allele using the primer PinA in BC_1F_1, **c** DNA amplification of *Pina-D1a* allele using the primer PinA in BC_2F_1 population, **d** DNA amplification of Pina-N1 in BC_1F_2 population. *L* ladder 1–24 wells represent the plants of the respective generations

Table 1 Generation-wise positive plants for puroindoline genes (*Pina-D1a*, *Pina-D1b*)

Generation	Total plants analyzed with marker	No. of plants with amplification of markers					
		PinA	Pina-N1	PinA + Pina-N1 (*Pina-D1a*/*Pina-D1b*)	PinA alone; no amplicon of Pina-N1 seen (*PinaD1a*/*PinaD1a*)	No. of plants on which background selection was exercised	No. of plants taken forward
F_1	105	105	99	99	6	–	99
BC_1F_1	400	215	nc	nc	–	112	19
BC_1F_2	98	52	75	29	23	nc	15
BC_2F_1	303	117	nc	nc	–	–	117

nc not checked, – not expected, () indicates putative genotype

close similarity with the recurrent DBW14. One hundred seventy-three polymorphic SSRs were run on these 112 BC_1F_1 plants, and only 19 BC_1F_1 plants were found to have high genome recovery of 80% and above. Now these 19 BC_1F_1 plants were bifurcated into two groups. In the first group, the 19 BC_1F_1 plants (4–5 spikes per plant) were backcrossed and in the second group same 19 BC_1F_1 plants (2 spikes per plant) were selfed to produce BC_2F_1 and BC_1F_2 generation, respectively. These 19 BC_1F_1 backcrossed plants produced 303 BC_2F_1 seeds. In the main season 2016–2017, 303 BC_2F_1 seeds were grown. All the plants were screened with PinA. One hundred seventeen plants were found to have *Pina-D1a* (Fig. 2c). These 117 BC_2F_1 were backcrossed to produce BC_3F_1 seeds, and simultaneously 2–3 spikes per plant were selfed to get BC_2F_2 seeds. Out of these 117 BC_2F_1 selfed plants, 31 BC_2F_1 selfed plants with high RPG % were harvested and its grain analysis was conducted. The seeds of BC_2F_2 borne on each of the plants were examined, each had both soft and hard grains, and therefore, the numbers were counted and are presented in Table 2. The 19 high RPG % BC_1F_2 plants seeds (selfed 19 BC_1F_1 plants) were also grown in the main season (2016–2017), and MAS was exercised with PinA and Pina-N1 markers in the 98 plants. DNA of 23 plants amplified PinA and did not amplify Pina-N1 marker, thus indicating that these were putative homozygotes for *Pina-D1a* (Fig. 2d). Further, these 23 plants were strictly screened for phenotypic similarity to the recurrent parent selected visually in the field and so 15 plants showing phenotypically similarity with recurrent parent were further taken forward. Rest eight plants showed less tillers, were shorter in height and therefore were discarded. The BC_1F_3 seeds from these 15 BC_1F_2 plants have been sown in off-season 2018 along with seeds from soft class of BC_2F_2.

Background selection

The parental polymorphism was carried out with 1110 SSR primers. Out of 1110 SSR primers used, only 173 SSRs were found to be polymorphic between DBW14 and Barham. The chromosome-wise distribution of the polymorphic markers is shown in Supplementary Table 3 and Supplementary Fig. S2. The individual SSR score for the respective polymorphic primers is given in Supplementary Table 4. These 173 primers were used for background selection. Some of them are depicted in Supplementary Fig. S1. The polymorphic markers were run on the BC_1F_1 plants, and RPG recovery was calculated. Nineteen BC_1F_1 plants with desirable foreground alleles and with RPG between 80 and 86.68% (Supplementary Table 2) were further backcrossed, and two spikes of each of these were selfed in off-season to obtain BC_2F_2 and BC_1F_2, respectively.

Recurrent parent genome recovery

One hundred seventy-three markers were used to analyze RPG recovery in the 112 BC_1F_1 plants carrying *PinaD1a* allele (see Supplementary Table 4). Chromosome 5D (carrying the *Pina-D1* gene locus) was found to have eight polymorphic SSRs between parents, viz., Xgwm205, Xbarc143, Xgwm174, Xgwm292, Xwmc215, Xbarc345, Xbarc140 and Xwmc765. Fourteen genotypes represented DBW14 allele of each of these primers. Similar exercise was conducted for other 20 other chromosomes. The % recovery based on these 173 SSRs ranged from 32.6 to 86.6%. The graphical genotyping (GGT) for chromosome 1A to 7D was carried out for the BC_1F_1 plants carrying *Pina-D1a* allele. The GGT for chromosome no. 5D is shown in Fig. 3. In Fig. 3, the red color bar segments represent the segments from donor parent Barham, while the blue color bar segment represents the genome segments from recurrent parent DBW14. More is the blue color, the more is the respective plant number close to its recurrent parent genome.

Table 2 Grain hardness observations in BC_2F_2 grains of the cross DBW14*3/Barham

Line number	No. of soft grains	No. of hard grains	Mottled	Hard + mottled	Total no. grains	GHI of soft class	GHI (SD)	TKW (g)	TKW (SD)	MOI (%)	MOI (SD)	DIA (mm)	DIA (SD)
16-92-7	317	275	33	308	625	28.8	8.1	42.1	7.3	11.4	0.1	2.8	0.2
16-86-6	328	182	24	206	534	25.7	10.9	33.7	4.6	11.6	0.2	2.7	0.3
16-75-2	341	364	60	424	765	32.3	9.0	39.9	4.5	11.3	0.1	2.7	0.2
16-126-6	207	110	68	178	385	35.5	14.8	36.4	8.3	11.3	0.2	2.8	0.4
16-128-5	374	291	0	291	665	20.7	10.1	39.6	7.8	11.3	0.1	2.8	0.4
16-86-7	100	165	52	217	317	22.0	17.0	35.7	5.8	10.9	0.2	2.7	0.3
16-148-1	242	353	5	358	600	28.8	11.2	28.6	5.0	11.4	0.3	2.5	0.2
16-70-4	125	125	25	150	275	12.1	9.3	30.9	5.7	11.6	0.6	2.5	0.4
16-125-8	520	180	0	180	700	18.2	10.2	40.4	6.8	11.4	0.2	2.9	0.2
16-125-4	224	189	1	190	414	34.1	9.2	40.7	4.7	11.7	0.1	3.0	0.2
16-125-10	137	181	7	188	325	35.0	8.6	29.7	6.1	11.5	0.1	2.6	0.2
16-69-4	108	250	92	342	450	31.8	7.9	37.1	5.5	11.2	0.2	2.8	0.2
16-142-6	186	250	14	264	450	25.8	12.2	39.5	8.2	11.2	0.2	2.8	0.3
16-149-10	72	129	96	225	297	25.1	10.1	38.8	5.0	11.1	0.2	3.0	0.3
16-138-4	310	343	77	420	730	32.8	12.2	34.9	5.7	11.5	0.2	2.8	0.3
16-113-1	850	300	0	300	1150	23.8	10.8	45.4	7.7	11.3	0.2	3.0	0.3
16-113-3	483	18	92	110	593	23.4	15.1	34.4	8.6	11.4	0.2	2.7	0.3
16-149-9	388	177	80	257	645	19.4	11.0	44.7	4.4	11.1	0.2	3.0	0.2
16-79-10	460	230	15	245	705	28.4	12.8	39.3	4.1	11.4	0.1	3.0	0.4
16-126-11	120	460	80	540	660	37.1	6.2	30.4	4.7	11.1	0.2	2.7	0.3
16-154-1	279	450	21	471	750	33.6	15.2	43.6	6.9	11.1	0.2	2.9	0.2
16-83-5	55	57	30	87	142	32.3	14.1	22.3	5.9	11.5	0.3	2.4	0.1
16-70-9	38	110	17	127	165	30.4	7.9	26.8	7.6	11.6	0.3	2.5	0.3
16-154-5	29	67	34	101	130	20.6	11.2	43.3	5.9	11.0	0.2	2.9	0.2
16-142-10	101	200	22	222	323	25.0	11.0	34.1	3.4	10.9	0.2	2.7	0.2
16-130-10	195	475	30	505	700	21.7	7.5	32.6	5.7	11.7	0.2	2.7	0.3
16-135-3	290	415	30	445	735	20.8	17.3	43.4	8.8	11.6	0.2	2.9	0.3
16-130-4	54	256	0	256	310	25.9	7.6	38.9	8.1	11.1	0.2	2.8	0.2
16-170-2	148	125	32	157	305	22.2	16.5	45.0	8.2	10.8	0.2	3.1	0.3
16-113-2	190	210	30	240	430	21.2	10.3	44.6	3.9	11.2	0.2	3.0	0.3
16-154-2	132	165	12	177	309	24.9	12.4	43.0	8.7	11.2	0.2	2.8	0.4
DP						26	21.3	26.3	8.3	14.3	0.5	2.3	0.2
RP						72.3	14.8	36.2	9.1	11.2	0.3	2.7	0.4

HI hardness index, *TKW* thousand kernel weight, *MOI* moisture %, *DIA* diameter

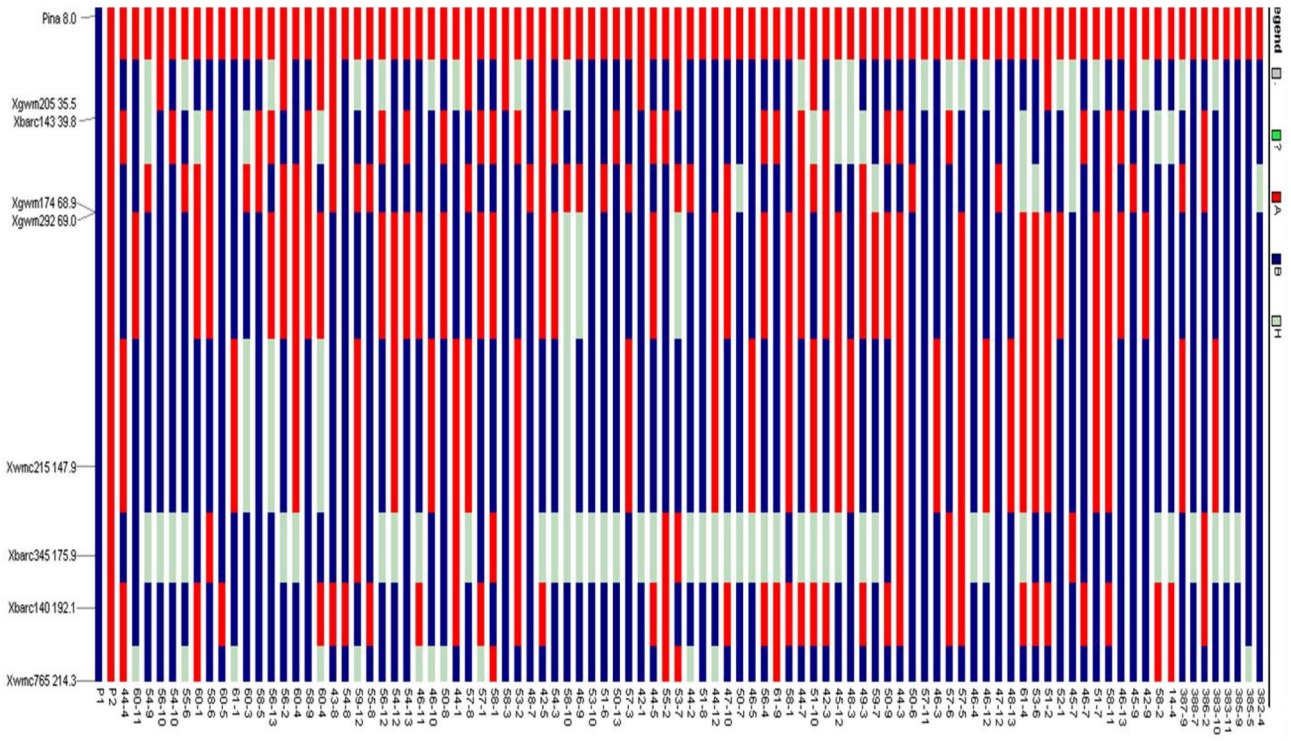

Fig. 3 Graphical genotyping of BC_1F_1 plants for chromosome number 5D (P1 = DBW14, P2 = Barham, red segments from donor, blue segments from recurrent parent, gray segments showing heterozygosity and green segments are those not amplified by SSR)

Fig. 4 Visual examination of the seeds of donor parent Barham, recurrent parent DBW14 and of their BC_2F_2 grains classified as hard, soft and mottled

Analysis of BC_2F_2 seeds for grain texture

The grain hardness data of the BC_2F_2 seeds are presented in Table 2. The grains from 31 plants and the donor parent and the recurrent parent were screened. Primary screening was based on the color and texture of the grain by the manual method. Grains were classified into hard and soft grain classes (Fig. 4). Further 30 grains from the putative soft category per segregating line were subjected to the SKCS (Table 2). The GHI in these grains ranged from 12.1 to 37.1. Line number 16-70-4 was observed to have the lowest GHI of 12.1, while line number 16-126-11 has the highest GHI of 37.1. TKW of soft grains ranged from 24.7 to 44.7 g and DIA from 2.8 to 3 mm.

Discussion

Marker-assisted selection (MAS) for transferring genes through plant breeding has been claimed to be cost- and time-effective. Foreground selection for a trait using molecular marker facilitates identification of positive plants for gene of interest at early plant stage, and thus, 50% population size is reduced in the breeding program enabling the breeders to decrease their efforts in handling of large populations (Collard and Mackill 2008). Molecular markers have also been acknowledged as a very powerful tool because of their reproducibility and precision (Gebremeskel et al. 2018; Gupta et al. 1999; Nadeem et al. 2018). One of the popular and successful schemes is

the marker-assisted backcross breeding which is used to improve many available good varieties which need one or a few simple inherited traits such as rust resistance or a quality trait to be added to them (Kolchanov et al. 2017). Molecular markers for quality traits in wheat have been slow to develop but in the last few years, their numbers have increased rapidly (Xu et al. 2013). Molecular maker-assisted breeding for agronomic and quality traits is now being deployed in many countries' national programs and private companies engaged in agriculture (Voosen 2009). De Bustos et al. (2001) carried out marker-assisted selection to improve HMW-GS in wheat. Similar, efforts were made by Vishwakarma et al. (2014) to introgress a high-quality protein Gpc-B1 in elite Indian variety. Reports on MAS for quality traits in wheat in India are relatively few (Gupta et al. 2008; Kumar et al. 2011; Tyagi et al. 2014). The knowledge on genes to improve end-use quality is diverse but its utilization in wheat breeding programs is way too less (Mondal et al. 2016).

In the present work, we have carried out MAS for transfer of grain softness allele to a hard-grained variety. In an earlier work (Rai et al. 2018), we have short-listed, based on an exhaustive quality and molecular analysis of genes for Puroindolines, HMW-GS and LMW-GS, three Indian wheat varieties out of the 30 analyzed, that could be improved for their texture and/or the gluten characteristics. One of them, DBW14 is grown in the region of northeastern plains zone (NEPZ). It is a hard-grained, awned variety. In the present work, MABB has been carried out in this variety to replace its *Pina-D1b* allele by *Pina-D1a* allele. Since it has *Pinb-D1a* allele, the resulting near-isogenic line (NIL) (*Pina-D1aPinbD1a*) is expected to be a soft-grained genotype. The donor awnless variety Barham has the alleles *Pina-D1aPinbD1a. PinaD1a* (amplified by PinA primer), *Pina-D1b* (amplified by Pina-N1 primer) were used for screening genotypes in F_1, BC_1F_1, BC_1F_2 and BC_2F_1 plants (shown in Fig. 2a–d). The BC_1F_1 and BC_2F_1 plants were screened with PinA (Fig. 2b, c) while the F_1 and BC_1F_2 plants were screened with Pina-N1 (for *Pina-D1b*) allele (Fig. 2d) in addition to the former marker. Along with the FG alleles, BG selection process was also practiced to transfer targeted genes to get NILs of DBW14. It was observed that even in individuals with high RPG recovery, the phenotypic similarity to the recurrent parent was less than optimum in several genotypes. Therefore, in BC_1F_1 and BC_1F_2 plants many of the genotypes that had positive target alleles by foreground selection were awnless and therefore, had to be rejected (Table 2). Similarly, many background-selected genotypes were lower tillering and much taller in height as compared to the RP and were therefore rejected despite having desired target alleles. Phenotypic selection could not be entirely substituted by background selection. A reason for this could be that only 173 polymorphic markers between parents were used for BG selection in BC_1F_1 generation. Given the size of wheat genome, these appear to be of limited value in selection of plants with a phenotype very similar to RP. Thus, combining phenotypic and genotypic selection in an MABB procedure is recommended. Similar experiment was designed by Zhou et al. (2003) which also conducted MAS combined with phenotypic screening in wheat. Another recent study by Kumar et al. (2018) demonstrated the synergistic effects of phenotyping along with genotyping done in the early generations on selection of better progenies. Plants with RPG ranging between 80 and 86.6% (Supplementary Table 2) were backcrossed or selfed further. The BC_1F_3 and BC_2F_2 seeds have been produced by shuttling the breeding materials between the main and the off-seasons per year for 2 years as shown in Fig. 1.

The seeds borne on BC_2F_1 (i.e., the BC_2F_2) were analyzed for their appearance and HI. Soft-looking, hard-looking and several mottled types of grains were observed. Selfing of a BC_2F_1 plant (*PinaD1aPinbD1a/PinaD1bPinbD1a*) should produce three types of zygotes: *PinaD1aPinbD1a/PinaD1aPinbD1a*, *PinaD1aPinbD1a/PinaD1bPinbD1a* and *PinaD1bPinbD1a/PinaD1bPinbD1a* in 1:2:1 ratio. Grain softness has been reported to be dominant over the hardness trait (Doekes and Belderok 1976; Guzmán et al. 2012); however, a mutation in any of the genes *PinaD1* or *PinbD1* produces a hard grain (Giroux and Morris 1997); therefore, the two classes in this case, viz., soft and hard grains in the ratio of 1:3, should be observed. However, it was found that the number of soft and hard grains did not fit into a 1:3 distribution; rather, no ratio fitted the soft and hard grains produced on different plants. When two laboratory personnel were asked one by one to classify these grains, they classified the grain differently as several grains were difficult to classify based on their appearance. There were also cases where the grains were mottled and appeared to have a yellow berry kind of appearance (Fig. 4). Yellow berry is a phenomenon commonly observed in durum grains and is said to be due to non-uniform deposition of protein in the grain (Headden 1915). The grains with such appearance were all hard as judged by crushing under teeth. Thus, on visual basis, it was found difficult to classify the seeds borne on a heterozygote.

Puroindoline proteins coded by the genes *PinaD1a* and *PinbD1a* have been shown to be exclusively produced in the starch cells of the endosperm (Wiley et al. 2007). It is stated that the *Pin* genes have tissue-specific promoters and are expressed in the endosperm cells during development of the grain. The endosperm is formed by double fertilization (primary endosperm nucleus fuses with one male gamete to give secondary nucleus while the other male gamete fuses with the egg cell) occurring in the megagametophyte. Therefore, each of the endosperm cells is 3*n* and has three alleles of each gene, two contributed by the female and one by the male gametophyte. Thus, the composition

of endosperm upon the selfing of a plant heterozygous for *PinaD1* gene, i.e., *PinaD1aPinaD1b* could be *PinaD1aPinaD1aPinaD1a* (referred to as type 1) or *PinaD1aPinaD1aPinaD1b* (referred to as type 2) or *PinaD1aPinaD1bPinaD1b* (referred to as type 3) or *PinaD1bPinaD1bPinaD1b* (referred to as type 4 here) depending upon the male and female gametes' constitution. In an endosperm type 1, three doses of allele '*a*' are present making threefold the protein (PINA) as compared to the other classes where twofold, onefold or no copies of protein may be present, respectively. Since *PinaD1a*-coded protein (*PINA*) physically binds along with the *PinbD1a* protein (*PINB*) to the lipids present on the starch cells of the endosperm (Kim et al. 2012; Pauly et al. 2013) giving rise to a soft endosperm texture, variation in the amount of *PINA* may influence the level of softness and thus, a reduction in softness of the level *PinaD1aPinaD1aPinaD1a* > *PinaD1aPinaD1aPinaD1b* > *PinaD1aPinaD1bPinaD1b* > *PinaD1bPinaD1bPinaD1b* is expected to appear. This may explain the reason for appearance of BC_2F_2 grains with varying degrees of hardness leading to confusion in their classification. There may be other reasons for varying degrees of hardness in the grains of different BC_2F_1 plants' harvest. The fact that GH score of soft seeds borne on each of the 31 BC_2F_1 plants varied from 12.1 to 37.1 and also the fact that the cross produced soft grains in some BC_1F_1 with HI much less than that of soft-grained parent Barham imply that additional genes influencing grain texture are present in both the hard- and soft-grained parents.

Thus, in the present work, the allele *Pina-D1a* was successfully transferred using molecular marker-assisted selection in the hard-grained genotype DBW14. The presence of soft grains and hardness index varying between 12.1 and 37.1 among the BC_2F_2 grains provide proof of the presence of *PinaD1a* allele in the BC_1F_1 plants amplifying the PinA primer.

Conclusion

It has been shown in the above study that it is possible to utilize molecular markers for transfer of genes or their alleles governing major quality traits in wheat. Marker-assisted backcross breeding could be successfully utilized for developing wheat lines where the *Pina-D1b* allele could be replaced by the *Pina-D1a* allele using an allele-specific PCR-based primer. After selfing of the heterozygous plants, the homozygotes could be selected by combining the foreground selection with the marker for *PinaD1a* and negative selection with the marker for *PinaD1b*. Background selection with molecular markers has resulted in plants in early backcross generations with greater similarity to the recurrent parent. We are now multiplying the selected homozygotes (*PinaD1aPinbD1a/PinaD1aPinbD1a*) for carrying out yield and quality analysis.

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Author contributions AMS was involved in planning and supervision of experiments, mentoring, financial facilitation and editing of manuscript. AR contributed to execution of experiments, molecular breeding and analysis of data and drafting of the manuscript. KR created F_1's and edited the manuscript. PS and TJK ran molecular markers (SSRs) in BC progenies. AKA contributed to grain hardness analysis. SKS, DG, MS and RBS were involved in field work in main and off-seasons. All the authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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