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RESEARCH ARTICLE

Validation and Marker-Assisted Selection of Stem Rust Resistance Gene *Sr2* in Indian Wheat Using Gel-Based and Gel-Free Methods

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Abstract

Stem rust resistance gene Sr2 is an important slow-rusting gene which has provided resistance against stem rust for many years. The *Sr2* gene has durable resistance against all stem rust pathogens including the Ug99 group of races. It shows recessive inheritance and is linked with two phenotypic markers, Pseudo Black Chaff (PBC) and High Temperature-Induced Seedling Chlorosis (HTISC). However, direct screening as well as phenotypic marker-based screening for selection of *Sr2* is difficult due to the effect of environmental factors and genetic background. Microsatellite marker *Xgwm533* linked to *Sr2* gene is useful for rapid screening of large populations. Here we report validation and use of *Xgwm533* for screening 21 wheat lines and 24 wheat genotypes for the presence of the *Sr2* gene. Furthermore, we also report the presence of a new, smaller allele (97 bp) of *Xgwm533* locus in a few genotypes that was also confirmed by sequencing. We also demonstrate a new SYBR green dye, melt-curve/profile-based assay for convenient screening of the *Xgwm533* locus, where the presence of different alleles can be differentiated in a gel-free manner.

Key words : Stem rust, Ug99, *Sr2*gene, marker-assisted selection, simple sequence repeat

Introduction

Stem rust of wheat, caused by *Puccinia graminis* f.sp. *tritici*, is one of the major diseases that can result in 100% yield loss. By deploying a major stem rust resistance (*Sr*) gene outbreaks of this disease has been kept under control. However, with the emergence of new, potent pathotypes like Ug99 and its derivative races, which overcame resistance conferred by major *Sr* genes (*Sr31, Sr24,* etc.), there is a serious challenge to prevent the damage due to stem rust of wheat (Pretorius et al. 2000).

Stem rust resistance gene *Sr2* is a race non-specific minor resistance gene transferred from YaroslavSchrank ex Schübler (*Triticum turgidum* L. ssp. *dicoccum*) to *Hope* cultivar (McFadden 1930). It has been in use for more than 60 years in breeding for achieving durable and broad spectrum adult plant resistance. It confers partial but durable resistance to all known stem rust races including the Ug99 group of pathotypes (Yu et al. 2017). Resistance conferred by *Sr2* is characterized by slow rusting phenotype and variable disease symptoms. In addition, *Sr2* gene resistance is characterized by the absence of a hypersensitive response which is a common characteristic of a major *Sr* gene (McIntosh et al. 1995a, b; Sunderwirth et al. 1980; Sunderwirth and Roelfs 1980). The *Sr2* gene has been widely used by CIMMYT and other countries across the world in conjunction with major *Sr* genes (Hayden et al. 2004; Rajaram et al. 1988). In India, *Sr2* has been deployed in combination with *Sr9*, *Sr11*, and *Sr31* to achieve durable stem rust resistance in wheat (Malik et al. 2013).

The *Sr2* gene is located on the short arm of chromosome 3B and inherited in a recessive manner (Hare and McIntosh

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1979a). Furthermore, its phenotype which can be identified only at the adult plant stage is significantly affected by presence of other *Sr* genes and environment variables like temperature, thus making its selection difficult in breeding programs (Brown 1997 McIntosh et al. 1995b). The *Sr2* gene is linked with two phenotypic markers viz. Pseudo-black chaff (PBC) and high temperature-induced seedling chlorosis (HTISC) (Eagles et al. 2001). However, both of these phenotypic markers are not ideal for selection with accuracy in large populations. For example, PBC expression, which develops on glumes and internodes is affected by the environment and also has a yield penalty. On the other hand, HTISC is recessively inherited and hence is difficult to select (Bhowal and Narkhede 1981; Hare and McIntosh 1979a, b). Spielmeyer et al. (2003) identified Simple Sequence Repeat (SSR) marker *Xgwm533* linked to the *Sr2* gene in a mapping population derived from a cross of Chinese spring (CS) X Chinese spring Hope substitution line for 3B (CS/Hope 3B). SSR locus *Xgwm533* amplifies a 120 bp allele in *Sr2* carriers, and a 155 bp (or higher size allele) or null allele in non-carriers. However, some Australian varieties like *Aroona*, that were known to be non-carriers of the *Sr2* gene also showed a 120 bp allele with *Xgwm533* marker, suggesting the existence of allelic homoplasy (same sized amplicon but different sequence) at the *Xgwm533* locus (Hayden et al. 2004).

Owing to this ambiguity in screening with *Xgwm533* many improvements have been reported. (Hayden et al. 2004) showed the allelic homoplasy by sequencing the 120 bp amplicons and designed a new set of SSR primers for differentiating *Sr2* carrier and non-carrier Southern Australian genotypes with more accuracy. In addition, the study also discovered an additional polymorphism in the *Xgwm533* locus, which may lead to false positives. Later on, (McNeil et al. 2008) used Bacterial Artificial Chromosome (BAC) contigs to develop a marker linked more tightly to the *Sr2* gene than the *Xgwm533*. Utilizing sequence information of the *Xgwm533* locus, (Mago et al. 2011) developed a Cleaved Amplified Polymorphic Sequence (CAPS) marker for more accurate screening of *Sr2* carrier and non-carrier genotypes.

In India (Malik et al. 2013), performed a comprehensive characterization of the *Sr2* gene in Indian wheat varieties using *Xgwm533* SSR markers and *csSr2* CAPS markers. Using data from both methods they could ascertain the presence of *Sr2* in 92% of Indian wheat varieties. We validated the *Xgwm533* marker in 13 wheat lines and screened for the *Sr2* gene in 10 varieties as well as 21 advanced breeding lines derived from different crosses at the Agriculture Research Station (ARS) Niphad. In addition, the *Xgwm533* marker was screened in 10 wheat varieties using SYBR green dye-based melt-curve method, in a gel-free assay. Here we report the presence of a new smaller (97 bp) *Sr2* linked allele in Indian wheat variety PBW-343 and an induced mutant derived from PBW-343. The smaller allele was sequenced to identify the deletion in the *Xgwm533* locus. The 97 bp allele also showed a different melt-profile than the 120 bp allele, and hence the method can be effectively utilized or screened in a gel-free manner.

Material and Methods

Plant Material

For validation of the *Xgwm533* marker, 13 wheat genotypes were used (Table 1). For screening the *Sr2* gene 10, wheat genotypes and 21 wheat lines derived from different crosses at ARS Niphad were used (Tables 2a and 2b). Genotypes CZ-56, HS-240, and NZ-164 were obtained from IARI Regional Station Wellington; NIAW-917, HI-8715, HI-8722, VL-941, HS-545, MACS-3828, WHD-948, HI-8703, UAS-320 from ARS Niphad; PBW-343, PBW-373 from PAU Ludhiana; Local Wheat Hango (LWH), Agra Local from IIWBR RS Shimla; Kalyansona, Sonalika from IARI New Delhi; TWM-97 was developed at Bhabha Atomic Research Centre, Trombay, Mumbai, India.

Genomic DNA Extraction

Genomic DNA extraction was done as per protocol detailed in (Eswaran et al. 2004). In brief, 200 mg leaf tissue was homogenized in 1.0 ml DNA extraction buffer (100 mM Tris, 20 mM EDTA, 0.5 M NaCl, 7 M Urea, 0.1% β-mercaptoethanol and 2% SDS). Phenol:chloroform:isoamyl alcohol (25:24:1) extraction was carried out and upper aqueous phase was collected. The aqueous phase was then extracted with equal volumes of chloroform:isoamylalcohol (24:1), gently mixed, and centrifuged at 12,000 rpm (10 min). DNA was precipitated by adding 0.1 volume of sodium acetate (3 M) and 0.7 volume of isopropanol. Precipitated genomic DNA was washed with 70% ethanol (twice) followed by

Table 1. Results of validation of *Sr2* gene in Indian wheat varieties using SSR marker *Xgwm533*.

Sr No	Name of Variety	*Sr2* status as per published report	*Xgwm533* allele observed (bp)
1.	CZ 56	+	120
2.	NIAW 917	+	120
3.	HW 3094	+	120
4.	PBW 343	+	97
5.	TWM 97	+	97
6.	PBW 373	+	120
7.	Kalyansona (KS)	+	120
8.	Sonalika (SK)	+	120
9.	Kite	-	Null
10.	Local Wheat Hango (LWH)	-	Null
11.	UC-306	-	Null
12.	Agra Local (AL)	-	155
13.	NZ 164	+	155
14.	HS 240	+	Null

Table 2a. Results of screening of Indian wheat genotypes and breeding population by *Xgwm533*.

Sr No	Genotype	*Xgwm533* amplicon observed (bp)	*Sr2* probable status
1.	VL-941	120	*Sr2* +
2.	HS-545	120	*Sr2* +
3.	UAS-320	120	*Sr2* +
4.	HD-2932	120	*Sr2* +
5.	HI-8715	300	*Sr2* -
6.	HI-8722	300	*Sr2* -
7.	MACS-3828	300	*Sr2* -
8.	WHD-948	300	*Sr2* -
9.	HI-8703	Null	*Sr2* -
10.	C-306	Null	*Sr2* -

Table 2b. Results of screening of advance wheat lines by *Xgwm533* screening.

Sr No	Wheat Lines	*Xgwm533* amplicon observed (bp)	*Sr2* probable status
1.	F_5 NIAW 917 X MP 4080	120	*Sr2* +
2.	F_5 NIAW 917 X MP 4080	120	*Sr2* +
3.	F_5 NIAW 917 X RAJ 4083	120	*Sr2* +
4.	F_5 NIAW 917 X RAJ 4083	120	*Sr2* +
5.	F_5 NIAW 917 X DBW 14	120	*Sr2* +
6.	F_5 NIAW 917 X DBW 14	120	*Sr2* +
7.	F_5 RAJ 4083 X DBW 14	120	*Sr2* +
8.	F_5 RAJ 4083 X DBW 14	120	*Sr2* +
9.	F_5 DBW 14 X RAJ 4083	Null	*Sr2* -
10.	F_5 DBW 14 X RAJ 4083	120	*Sr2* +
11.	F_5 RAJ 4083 X KING BIRD	Null	*Sr2* -
12.	F_5 RAJ 4083 X KING BIRD	120	*Sr2* +
13.	F_5 NIAW 34 X RAJ 4083	120	*Sr2* +
14.	F_5 NIAW 34 X RAJ 4083	120	*Sr2* +
15.	F_5 NIAW 917 X DBW 14	Null	*Sr2* -
16.	F_5 NIAW 917 X DBW 14	Null	*Sr2* -
17.	F_5 RAJ 4083 X DBW 14	Null	*Sr2* -
18.	F_5 RAJ 4083 X DBW 14	Null	*Sr2* -
19.	F_5 DBW 14 X RAJ 4083	120	*Sr2* +
20.	F_5 DBW 14 X RAJ 4083	Null	*Sr2* -
21.	F_5 NIAW 34 X RAJ 4083	120	*Sr2* +

absolute ethanol (once), and dissolved in Tris-EDTA buffer (Tris-Cl:10 mM, EDTA: 1 mM, pH: 8.0). DNA preparations were treated with RNAse (10 mg/sample) at 37°C. Quantity of the DNA samples was an estimated quantity (by measuring OD_{260} on a spectrophotometer (UV-1800, Shimadzu, Japan), while integrity (extent of shearing) was evaluated on 0.8% agarose gel.

Polymerase Chain Reaction

PCR amplification of *Xgwm533* marker was performed on Mastercycler gradient PCR machine (Eppendorf, Germany). Reaction components for PCR were procured from Bangalore Genei (India). The assay volume (25 µl) contained genomic DNA (50-100 ng), dNTPs (250 µM each), 2.5 µl 10X reaction buffer (15 mM Tris-Cl pH 9.0, 50 mM KCl, 0.01% gelatin), 3-5 picomole of each primer, $MgCl_2$ (2.0-3.5mM), and 1.0 unit of *Taq* DNA polymerase. Thermal cycling conditions for PCR amplification were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C, annealing at 60°C and extension at 72°C, and a final extension of 2 min at 72°C at the end.

Agarose Gel Analysis

PCR amplified products were analyzed on 2.5% agarose gel (Sigma-Aldrich, USA) by electrophoresis (8-10 V cm^{-1}) in 1X TBE buffer. The DNA fragments were stained with ethidium bromide and photographed under UV light on a gel-documentation system from Syngene (Syngene, UK). Sizes of DNA amplicon were estimated using 100 and 20 bp DNA ladders (Sigma-Aldrich, USA).

SYBR Green-based, melt-curve analysis

For detection of amplified microsatellite markers without running a gel, SYBR Green (S9430, Sigma-Aldrich, USA), a non-specific DNA binding fluorescent dye was added to the PCR assay mix (described above) at 1X final concentration. These products were analyzed using melt-profiling by measuring the fluorescence intensity of products on an Eppendorf Mastercycler realplex^4 machine in a continuous mode while the temperature was raised from 60 to 95°C (3.5°C min^{-1}) (Vishwakarma et al. 2016). The fluorescence data was transformed to the negative first derivative using the Eppendorf Mastercycler ep realplex software (version 2.2).

Cloning and sequencing of alleles amplified from *Xgwm533* locus

The two *Sr2* linked amplicons, i.e. 120 and 97 bp sized SSR alleles were purified from PCR mix. In brief, the total volume of PCR master mix was increased to 500 µl by adding water and an equal volume of PCI (25:24:1) was added to collect the aqueous phase. Other steps were carried out as per genomic DNA extraction method described above, however, after chloroform:isoamylalcohol extraction the precipitation was carried out in 100% ethanol. PCR products were precipitated and dissolved in nuclease-free water and ligated to the linearized pXcmkn12 TA vector. The ligated products were electroporated into *E. coli* DH5α strain, then the recombinant clones were selected by plating on an LB-Agar plate containing ampicillin. The presence of the insert (*Xgwm533* alleles) in clones was confirmed by colony PCR using a gene-specific and M13 primer combination. The positive clones were sequenced using the Sanger

sequencing method. The web-based Clustal Omega tool using default settings was used to perform multiple sequence alignment of the sequences of alleles of the *Xgwm533* SSR marker.

Result

Validation and screening of *Sr2* using *Xgwm533* marker

The SSR marker *Xgwm533* was validated in 14 wheat genotypes (Table 1). Results showed that in wheat genotypes known to carry the *Sr*2 gene (based on published reports), the marker amplified a band of 120 bp. However, in wheat genotypes PBW-343 and TWM-97 a shorter allele of 97 bp was observed (Fig. 1). In *Sr2* non-carrier wheat genotypes either a null allele or a 155 bp allele was observed. Of 14 genotypes HS-240 and NZ-164 did not amplify the expected amplicon (120 bp allele) for an *Sr2* carrier genotype. Genotypes HS-240 and NZ-164 (both reported to carry the *Sr2* gene) amplified the null and 155 bp alleles, respectively. Among the 10 wheat genotypes screened for the *Sr2* gene, four amplified 120 bp alleles (indicating the presence of the *Sr2*gene), two showed null allele, and the remaining four showed 300 bp allele indicating the absence of *Sr2* gene (Table 2, Fig. 2a). The marker was also used for evaluating *Sr2* status in wheat breeding lines. Of the 21 wheat advanced lines, 14 showed 120 bp amplicon (indicating presence of *Sr2* gene), while seven showed null allele (Table 2b, Fig. 2b).

Identification of new allele at *Xgwm533* locus

We observed that in the Indian wheat variety PBW-343, the *Xgwm533* locus amplified 97 bp allele instead of the most common 120 bp allele (Fig. 1). PBW-343 is reported to carry the *Sr2* gene, suggesting that the new allele is linked to the *Sr2* gene. In addition, a gamma ray-induced mutant TWM-97 derived from PBW-343 at the Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai, India, also showed 97 bp allele.

Sequencing of *Xgwm533* locus

DNA Sequencing of the characteristic 120 bp allele and the newly found 97 bp allele showed that the true length of the 120 bp allele was 117 bp. It was also observed that there is a 24 bp deletion at position 52 as well as an insertion of 3 bp at position 86 in the amplicon from TWM-97 which

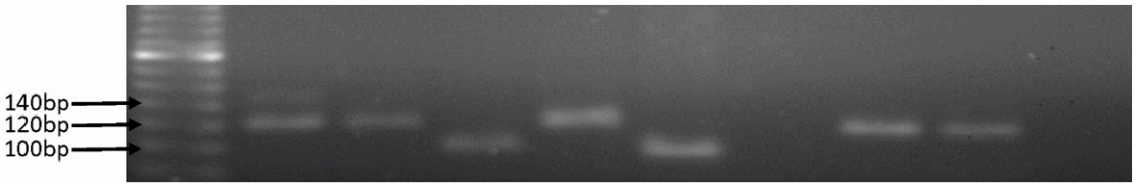

Fig. 1. Validation of *Xgwm533* marker for screening of *Sr2* genes in representative wheat varieties. Lane 1: 20 bp DNA ladder, Lane 2: CZ-56, Lane 3: NIAW-917, Lane 4: PBW-343, Lane 5: PBW-373, Lane 6: TWM-97, Lane 7: HS-240, Lane 8: Kalyansona, Lane 9: Sonalika, Lane 10: Local Wheat Hango.

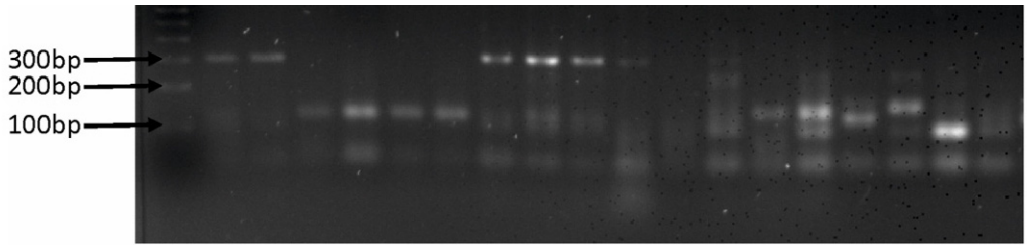

Fig. 2a. PCR amplification of Xgwm533 allele's representative wheat genotypes. Lane 1: 100bp marker, Lane 2-3: HI-8722, Lane 4-5: VL-941, Lane 6-7: HS-545, Lane 8-9: MACS-3828, Lane 10-11: WHD-948, Lane 12-13: HI-8703, Lane 14-15: UAS-320. Lane 16: CZ-56, Lane 17: NZ-164, Lane 18: PBW-343, Lane 19: Local Wheat Hango.

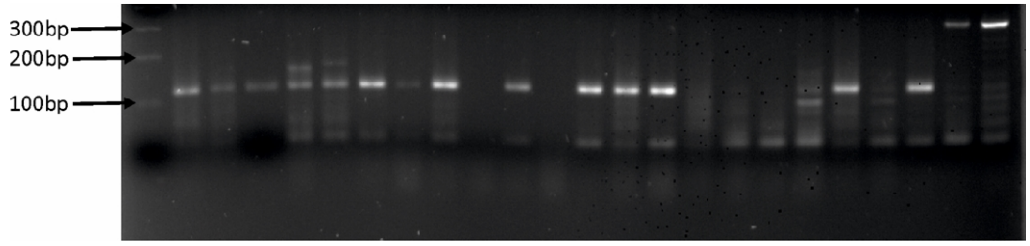

Fig. 2b. PCR amplification of *Xgwm533* marker for marker-assisted selection of representative advance lines. Lane 1: 100 bp marker, Lane 2 to Lane 22: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21. (Wheat lines serial no. as per Table 2b). Lane 23 and Lane 24: HI-8715.

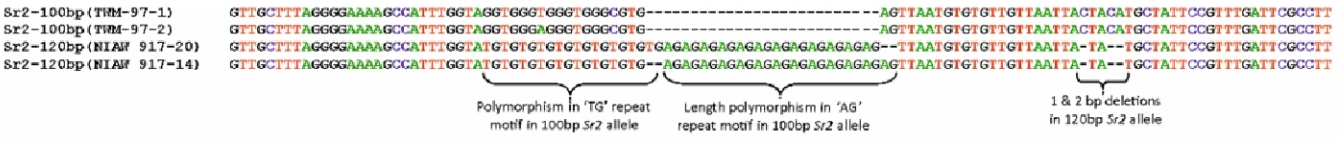

Fig. 3. Multiple sequence alignment showing the regions of length and sequence variability in 100 and 120 bp alleles of *Xgwm533* locus.

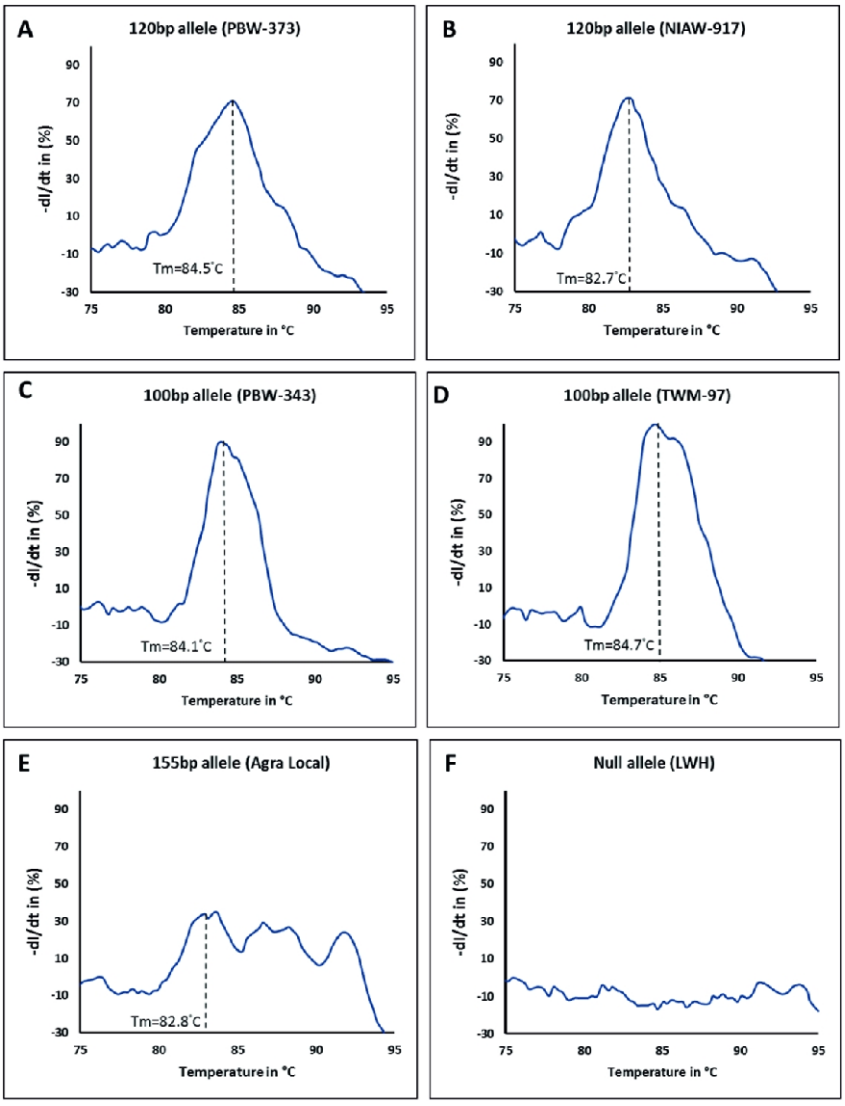

Fig. 4. SYBR green dye based melt-curve analysis of alleles of *Xgwm533* locus. T_m values of *Xgwm533* alleles: PBW-373: 84.5 °C, NIAW-917: 82.7°C, PBW-343: 84.1 °C, TWM-97: 84.7 °C, Agra Local: 82.8 °C.

resulted in a 97 bp allele locus (Fig. 3). In addition, there was considerable sequence variation. The 117 bp allele has a GC content of 41%, while the 97 bp allele had a GC content of 45% which may be responsible for its different melting temperature (T_m) in the melt-curve profile analysis. In addition, it was also found that there is considerable allelic homoplasy (i.e. same size but different sequence) which leads to different GC% of the amplicons. This difference was also evident in the T_m values obtained during the melt-curves of genotypes.

Melt-curve analysis of different alleles of *Xgwm533* locus

All the four alleles viz: 120, 97, and 155 bp, and null (no amplicon) gave specific and clean melt-curves and could be easily screened using gel-free melt-profiling method (Fig. 4). The T_m of these alleles were as follows: 1) 120 bp (NIAW-917): T_m82.7°C, 2) 120 bp (PBW-373): T_m 84.5°C, 3) 97 bp (PBW-343): T_m84.1°C, 4) 97 bp (TWM-97): T_m 84.7°C, 5) 155 bp (Agra Local): T_m82.8, and 83.6°C. Null allele (Local Wheat Hango) did not show any characteristic melt-curve (and no Tm) due to the absence of amplicon. As

the T_m the various alleles differed the melt-profile helped inaccurate determination of particular alleles in a convenient and time-saving, gel-free assay.

Discussion

Sr2 is an important stem rust resistance gene providing durable resistance against all prevalent stem rust races including the Ug99 group of races (Singh et al. 2011). Due to its importance for resistance against stem rust, *Sr2* has been widely deployed with other major genes in world as well as CIMMYT accessions (Rajaram et al. 1988). Selection of *Sr2* gene is difficult due its expression only in mature adult plants and significant effect of background, presence of other *Sr* genes and environment (Brown 1997). Although phenotypic marker like PBC and HTICS are linked to *Sr2* selection of *Sr2* using these markers is difficult (Bhowal and Narkhede 1981; Hare and McIntosh 1979b). Marker-assisted selection is a convenient method for such cases where direct resistance screening is difficult. *Xgwm533* was found to be tightly linked to *Sr2* gene and has been widely used for its selection (Spielmeyer et al. 2003).

We here utilized *Xgwm533* marker for validation in 14 wheat genotypes and screening in 10 genotypes as well as 21 advance wheat lines. The *Xgwm533* marker could discriminate between *Sr2* carriers and non-carriers based on amplicon amplified except in two genotypes. The *Xgwm533* marker as found in our study and in previously reported studies cannot accurately determine *Sr2* status in some wheat genotypes and hence screening for *Sr2* genes should be carried out with a combination of markers (Malik et al. 2013). Several suggestion for improvement in marker-assisted selection for *Sr2* gene, e.g. Mago et al. (2011) suggested gene driven marker for *Sr2* on lines with *Lr34/Yr18* marker.

We also report a new 97 bp allele in two genotypes PBW-343 and TWM-97, suggesting that the *Xgwm533* locus is highly polymorphic. In addition, based on sequencing data it is concluded that there is significant allelic homoplasy at the *Xgwm533* allele locus which is evident in the T_m of different alleles (Fig. 4). We utilized SYBR green-based, low-resolution melt curve analysis for screening of *Sr2* alleles. We could successfully screen these allele using melt curve assay and discriminate the different alleles in a gel free and high-throughput manner (Vishwakarma et al. 2016).

Conclusion

Marker-assisted selection using *Xgwm533* or other improved markers can help in accurate screening of the *Sr2* gene which is highly important especially in the current scenario where new pathogens are overcoming the major resistance gene. In addition, the use of high throughput methods like the melt curve assay can save time and cost of such analysis and as a result may be a complement in breeding efforts.

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