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Research Article

Marker-trait association for fertility restoration using *Rf8* gene in *Triticum timopheevii* based male sterile cytoplasm of wheat

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Abstract

Heterosis is an important way to improve yield and quality for wheat. Effective restoration of fertility, its genetic control however remains elusive. Among 9 reported fertility restorer genes for *Triticum timopheevii* cytoplasm, *Rf8* has been mapped on chromosome 2DS. Two F2populations from crosses,CMS BWL-5203/R-6 and PHW-1 were grown in 2018 off-season at Punjab Agricultural University Regional Research Station, Keylong (H.P) to study the efficacy and robustness of markers linked with *Rf*8. Data was recorded on extent of fertility restoration and three linked markers namely *Xwmc503, Xgwm296* and *Xwmc112* were used for this study. Among these, *Xwmc503* present at 3.3 cM away from *Rf*8 showed significant association with fertility restoration in both the crosses. In contrast, marker *Xgwm296* linked at 5.8 cM did not reveal significant association in any population. However, the third marker *Xwmc112* did not get amplified in any of the populations. *Xwmc503* marker thus can potentially be useful for selecting restorers with *Rf8* gene which further could be used for transferring this very gene into potentially elite genotypes to enrich hybrid male parental pool.

Keywords: *Rf8*, fertility restoration, hybrid wheat, cytoplasmic genetic male sterility, SSR markers.

1. Introduction

Globally, wheat (*Triticum aestivum* L.) is the second most important crop after maize. Its contribution is appraised to 21% of food calories and 20% of protein to more than 4.5 billion people, especially in developing countries including India and China (Shewry and Hey 2015). Across the world, wheat is grown over 217 million hectares land during 2019-20 with overall production of about 764 million metric tonnes (USDA 2020). In wheat, the rate of yield gain has been reduced over the last decade. To meet out the projected demand of food production in near future, there is need to explore alternate approaches to surpass the yield barriers and make wheat cultivation more remunerative. In that scenario, one of the most feasible options is to reap the yield benefits from heterosis (Pickett 1993, Whitford *et al.,* 2013). Besides significant yield advantages, hybrids have also been reported to exhibit improved yield stability (Muhleisen *et al.,* 2014). Exploiting hybrid vigor in wheat through development of hybrids is considered promising, as it offers a mean of meeting global food demand due to yield heterosisin the wake of variable climatic changes in years to come (Boeven 2016). Hybrid wheat research is being re-initiated across wheat breeding groups in the world. With hybrid wheat consortia of public institutes in USA to private sector Bayer and Syngenta initiating research & development in the area. However, the major challenges for its success are improved restoration and cost of seed production with respect to purelines. Reduction in cost of hybrid seed production in turn depends upon development of an efficient system evoking out-crossing in wheat.

The highly self-pollinated nature of wheat plant with chasmogamous flower habit demands a shift in pollination system to facilitate heterosis breeding. That is to say the

paramount requirement to enhance natural out-crossing is by introducing male sterility. The theoretical idea of exploitation of heterosis in wheat was reported initially in 20th century by Freeman (1919) and Engledow and Pal (1934). There are many systems introduced for serving this purpose such as genetic male sterility (Pugsley and Oran 1959) and chemical induction (Striff *et al.,* 1997), thermo-photosensitive genetic male sterility (TPGMS) regulated by recessive nuclear gene (Zhang *et al.,* 2006). Another important system namely cytoplasmic genetic male sterility (CGMS) also reported in many crops controlled by nuclear-cytoplasmic interactions. However, its practical possibility was not worked out in wheat until the discovery of an effective cytoplasmic male sterility and fertility restoration system from related wild species such as *Ae. caudata* (Kihara 1951), which opened up new possibilities for commercial hybrid seed production in wheat. The discovery of male sterility and fertility restoration systems in the 1960s accelerated interest in hybrid wheat from both the public as well as private sector. Wilson and Ross (1962) and Schmidt *et al.,* (1962) independently introduced cytoplasmic male sterility in wheat variety 'Bison' by using then novel source, *T. timopheevii* at Kansas State University.

A genetic system for restoring F_1 hybrids fertility carrying *T. timopheevii* cytoplasm was first revealed by Wilson and Ross (1962). Most workers have concluded the restoration to be of dominant nature (Robertson and Curtis 1967).Difficulties in obtaining desirable restoration also arose from interactions of restorer genes with genetic background as well as environment. The complexity of the restorer system is evident from the fact that restorer genes (*Rfs*) are carried on many chromosomes viz. on chromosome 1, 2, 5, 6 and 7. Major factors responsible for low levels of fertility restoration include high and low temperature, moisture scarcity and restriction in plant development (Virmani and Edwards 1983).

Nine Rf genes have been reported till date to restore fertility against *T. timopheevii* cytoplasm (T-type), and their chromosomal locations have been determined as *Rf1* (1A) (Du *et al.,* 1991), *Rf2* (7D) (Bahl and Maan 1973, Maan *et al.,* 1984), *Rf3* (1B) (Tahir and Tsunewaki 1969, Zhou *et al*., 2005), *Rf4* (6B) (Maan *et al.,* 1984), *Rf5* (6D) (Bahl and Maan1973), *Rf6* (5D) (Bahl and Maan 1973), *Rf7* (7B) (Bahl and Maan 1973), *Rf8* (2D) (Sinha *et al.,* 2013) and *Rf9* (6A)(Shahinnia *et al.,* 2020). In addition to restorer loci, environmental factors (Johnson *et al.,* 1967) along with epistatic effects of genetic background (Maan *et al.,* 1984) also influence the fertility restoration. It was proposed that stacking of restorer and modifier loci could be beneficial to overcome such obstacles (Johnson and Patterson 1977). Restriction fragment length polymorphism (RFLP) markers were used by Ma *et al.,* (1995b) to map a gene *Rf6,* transferred from chromosome 6U of Ae. *umbellulata* into wheat, restoring fertility to *T. timopheevii* cytoplasm. A fertility restorer gene, *Rf3* was mapped by Geyer *et al.,* (2016a) between the marker loci *Xbarc128* and *Xwmc406* on chromosome 1BS. Another study by Geyer *et al.,* (2016b) showed that *Rf3* explains the restoration capacity of a large proportion of European common wheat lines, but additional modifier loci are needed for full restoration of male fertility by *Rf3* (Würschum *et al.,* 2017).

The *Rf8* gene identified and validated by Sinha *et al.,* (2013) is the only restorer gene reported on chromosome 2D so far. It is reported to impart good fertility restoration in hybrids and has unique location in the genome, making it a distinct gene to study its efficacy and inheritance pattern in the population harbouring this *Rf8*gene.In the present investigation, we aimed at examining the association of *Rf8*gene (2DS) markers with the fertility restoration in order to assess the efficacy of linked markers, as this major gene was studied by Sinha *et al.,* (2013) along with its identification and mapping.

2. Materials and methods

2.1. Plant Material

Two different crosses CMS BWL-5203/R-6 and PHW-1having 111 and 99 F2 plants were chosen respectively to study the association of markers linked to *Rf8* and the extent of its restoration ability. One cross (PHW-1) was known for carrying *Rf8* gene and other (CMS-BWL 5203/ R-6) we had chosen from in house hybrid material on assumption that the *Rf* gene is coming from *T. timopheevii.* So, there was good probability of it being similar or allelic.

2.2. Fertility evaluation and data analysis

The F2 population of 210 plants was grown at PAU offseason research station, Keylong (Lahaul&Spiti, Himachal Pradesh) in May 2018. The low relative humidity over there promotes cliestogamy thereby ensuring selfing and fertility restoration. For further precaution, the spikes were tagged and bagged on onset

of earing. Number of seeds formed per bagged spike was used to access the fertility restored in the plants.Leaf samples were taken for marker-trait association analysis at wheat lab, Department of Plant Breeding & Genetics, PAU (Ludhiana). Percent seed set per spike was calculated for defining three major classes for restoration ability viz. low, medium and high restoration.

Fertility restoration (%) = (Total no of seeds/spike)/(Total no of 1^0, 2^0 and 3^0 florets per spike)×100

(Tucker *et al* 2017)

Fertility restoration of 0-5% was classified as low, between 6-40% under medium restoration class and more than 40% was recognized under high restoration. The statistical analysis was done using R software (version 4.0.2).

2.3. DNA isolation and quantification

DNA was extracted using CTAB method (Murray and Thompson 1980). Young leaves were collected from 210 F2 plantsfrom 2 crosses. Tissuelyzer was used for the crushing of the dried leaf samples. Finally DNA samples were dissolved in 100 µl TE buffer and checked for quality and quantity using nanodrop spectrophotometer.

2.4. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed in ABI Thermo Fisher scientific thermocyclers by using 60 ng/µl genomic DNA/reaction. Targeted DNA regions were amplified using PCR (Table 1). The reaction volume of 10 µl containing the template gDNA (60ng/µl) along with all the other reaction components was used. The amplified product was resolved on 2.5% agarose. Along with the DNA samples, a 50 bp DNA ladder was also loaded to estimate the size of each DNA fragment amplified. The information on gene targeted in the current study along with their linked markers and their PCR amplification cycle is given in the Table 1.

Table 1. PCR amplification details for Rf8 linked SSRs used in the study along with their efficacy and permissible errors based on their genetic linkage with Rf8 (Sinha *et al*., 2013)

S.no.	SSR Marker	Marker efficacy (%)	Permissible errors (for 210 plants)*	Ta (0C)	No. of Cycles
1.	Xwmc503(3.3 cM)	96.7	14	60	40
2.	Xwmc296(5.8 cM)	94.2	24	55	45
3.	Xwmc112(6.7 cM)	93.3	28	61	40

*includes both the false positives and false negatives

3. Results and discussion

3.1 Fertility Restoration

Several investigations have been conducted so far in order to unravel the genetics of nuclear fertility restoration in hybrid wheat. Significant variations in previous studies' results suggest variability in the genetic control of the fertility restoration trait in wheat. Fertility restoration as supported by several studies seems to be monogenic (Hughes and Bodden 1977, Zhou *et al.,* 2005) or digenic trait (Schmidt and Johnson 1963). A single restorer locus does not yield a complete and stable fertility restoration in hybrids with *T. timopheevii* cytoplasm (Sage 1972).

The universal expression of as many *Rf* genes as possible seems to be beneficial for obtaining stable and high fertility restoration (Ma and Sorrells 1995a). Zhou *et al.,* (2005) successfully documented the three closely linked SSR markers for one of the robust fertility restoration genes, *Rf3* i.e. *Xbarc207, Xgwm131* and *Xbarc61,* on chromosome 1B.

Tomar *et al*., (2009) studied agro-morphological and molecular diversity among exotic and indigenous fertility restorers against *T. timopheevii* cytoplasm and reported that fertility restorers were genetically diverse. In addition to seven major genes, some minor QTLs involved in fertility restoration have also been reported on chromosomes 2A, 2B, 4B, 5A 6A and 7D (Ahmed *et al*., 2001, Zhou *et al*., 2005). Out of nine known fertility restorer genes

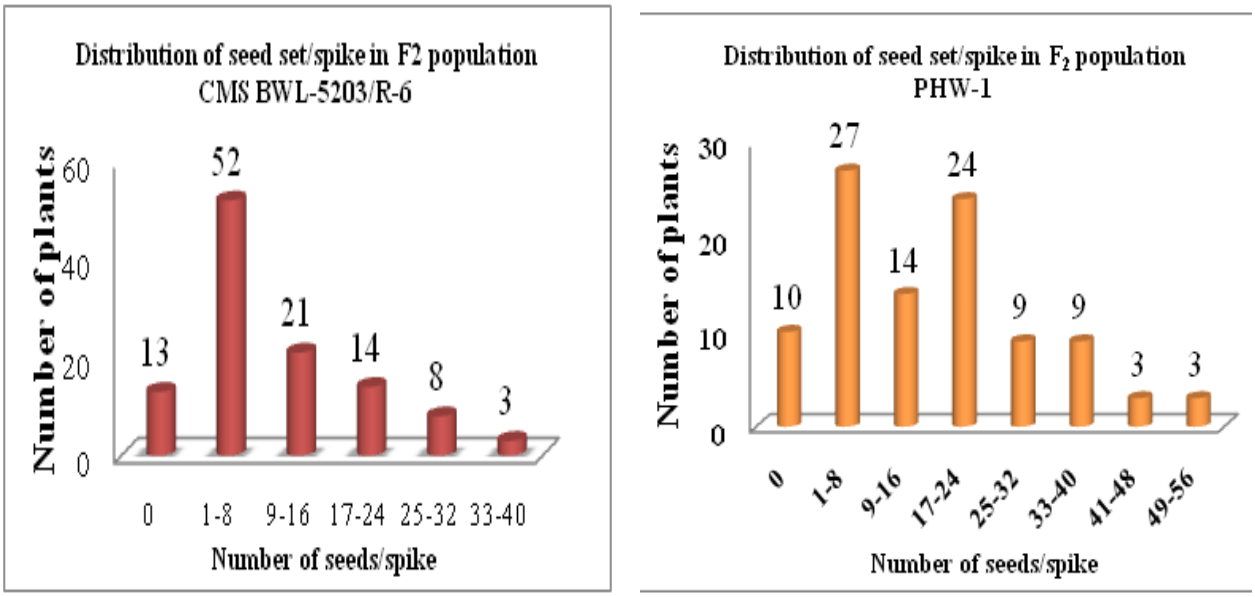

Fig.1. Frequency distribution in F2 population of two crosses CMS-BWL 5203/R-6 and PHW-1 respectively based on seed set per bagged spike

(Rf), only one gene Rf3 was localized with restriction fragment length polymorphism (RFLP) markers (Kojima *et al.*, 1997). Zhou *et al.*, (2005) identified the closely linked SSR markers, *Xbarc207, Xgwm131* and *Xbarc61* to the fertility restorer gene *Rf3* on chromosome 1B. In our study,seed set in the bagged spike was accounted for the fertility restoration character. After harvesting the matured spikes followed by their thrashing and counting of selfed seed obtained per bagged spike representing the single F2 individual.

There were 2 major phenotypic classes observed based on the number of seed set per spike in bagged F2 populations. The range obtained was 0-56 seeds/spike (Figure 1). The mean seed set for population comes out to be 10.8 seeds/spike and floret mean was 52.5 florets/spike. Further,with respect to seed set, plants with ≤ 4 seeds per spike were grouped as sterile to the fertile ones having ≥ 5 seeds per spike (Sinha *et al.*, 2013). As far as two F2 populations are concerned, both the populations have different pedigrees, indicating strong possibility of difference in genetic control of this very trait.

PHW-1 was known for Rf8 gene and other cross (CMS-

Table 2. 2-analysis for segregation in F2 population with respect to seed set per spike in cross-1 (CMS-BWL 5203/ R-6) (=0.01)

Class	Genotype	Expected ratio	Observed no of plants	Expected no of plants	2-value	P-value
Fertile (≥ 5seeds/spike)	RaRaRbRb* RaraRbRb RaRaRbrb RaraRbrb	9	51	63	4.78	0.028
Sterile (0-4 seeds/spike)	RaRarbrb raraRbRb Rararbrb raraRbrb rararbrb	7	60	48		
Total no of Plants			111	111		

*Ra: restorer locus 1, Rb: restorer locus 2

BWL 5203/R-6) was selected from our in house hybrid material at PAU on assumption that the Rf gene is inherited from *T. timopheevii.* Hence, there was decent possibility of the restorer gene being similar or allelic.As it was assumed in cross-1 (CMS-BWL 5203/ R-6) that the trait is controlled by two genes with complementary gene action. So, the null hypothesis is the fertility restoration is controlled by two genes having complementary action.

The ratio we observed from the F2 analysis fits well as 9:7 revealing complementary gene action of a 2 restorer loci in the CMS-BWL 5203/ R-6 population. Absence of at least one dominant allele at each of the loci will give rise to sterile ears i.e.0-4 seeds/spike set otherwise leading to fertile ears. As the p-value obtained is greater than level of significance i.e. 0.01, accepting null hypothesis that fertility restoration is a digenic trait exhibiting complementary gene action.

As for cross-2 (PHW-1)having to different pedigree than cross-1, it was assumed that the trait is controlled by a single gene with dominant action (null hypothesis).The ratio we observed from the F2 analysis (Table 3) fits well as 3:1 revealing dominant action of a single restorer locus in the PHW-1 population. Only recessive homozygotes allele (rr) will give rise to sterile ears i.e. 0-4 seeds/spike set otherwise exhibiting fertile ears. As the p-value obtained is far greater than level of significance i.e. 0.01, indicating that fertility restoration is a dominant trait controlled by single gene in cross-2. It is also quite likely that the fertility restoration can potentially be affected by environmental factors as well (Johnson *et al*., 1967, Sage 1976). In addition to that, some modifier loci and minor genes could also have an elusive role in the segregating population upon level of fertility restoration (Ali *et al*., 2011, Geyer *et al*., 2018). In ourstudy with 2 crosses, the results differ possibly due to wider differences in the parentage of the populations under investigation, hence having similar or allelic version of the *Rf8* restorer gene.

Table 3. 2-analysis for segregation in F2 population with respect to seed set per spike in cross-2 (PHW-1)(=0.01)

Class	Genotype	Expected Ratio	Observed no of Plants	Expected no of Plants	2-value	P-value
Fertile (≥ 5 seeds/spike)	RR	3	69	75	1.485	0.223
Sterile (0-4 seeds/spike)	rr	1	30	24		
Total no of Plants			99	99		

3.2 Association of fertility restoration with Rf8 markers

In a study done by Sinha *et al.,* (2013), *Rf8* was reported to be found on short arm of chromosome 2D, which is first ever finding of any *Rf* gene(s) on that location so far. Three closely linked markers to *Rf8* i.e. *Xwmc503*, *Xwmc296* and *Xwmc112* were validated in a set of restorer, cytoplasmic male sterile and maintainer lines. These three simple sequence repeat (SSR) markers closely linked to fertility restoring *Rf8* gene were used for genotyping the plants. Marker *Xwmc503* was found to be located on chromosome 2DS at a genetic distance of 3.3 cM and is the closet marker associated with the trait. While *Xgwm296* and *Xwmc112* are the flanking markers present on both sides of the gene at a distance of 5.8 cM and 6.7 cM. The LOD score values for these three markers are10.12, 8.58 and 7.17 respectively (Sinha *et al.,* 2013). On the basis of their reported genetic linkage with *Rf8,* the efficacy of each marker was approximated as 96.7% (*Xwmc503*), 94.2%(*Xgwm296*) and 93.3% (*Xwmc112*) respectively(Table 1).

In order to check out their extent of association with the trait, the null hypothesis (H0)formulated for this studywas both the fertility restoration and Rf8 markers are independent variables otherwise association between both the characters under study (H1).

Based on the percent seed set/spike, fertility restoration in plants with seed set/spike from 0-5% was classified as low restoration, between 6-40% under medium restoration classes and more than 40% was recognized as high restoration.

Table.4 and Table.5 show segregating allelic classes observed for both the *Rf8* linked markers under 3 distinguished levels of fertility restoration (% seed set in bagged spikes).

Hence, as depicted from thecombined 2-analysis of 2 linked SSR markers in table.6, it is clear thatforXwmc503, P-value is less than level of significance in both the crosses, rejecting the null hypothesis. Therefore, *Xwmc503* showed

Table 4. 2 - contingency table and analysis for fertility restoration reaction as well as allelic classes observed in cross-1 (CMS-BWL 5203/R-6) population

Restoration reaction	Allelic classes for *Xwmc503*			2-value	P-value
	RR	AR	NN	9.84	0.043*
Low (≤5%)	7	14	14		
Medium (6-40%)	24	32	17		
High (≥40%)	4	1	0		
	Allelic classes for *Xgwm296*			6.88	0.142
Low (≤5%)	3	33	15		
Medium (6-40%)	0	42	12		
High (≥40%)	0	6	0		

*significant at 0.05 probability level
R: restorer allele, A: sterility allele and N: nil allele (Figure 2)
RR- *Restorer homozygous parent*, AR- *Heterozygous parent* and NN- *Nil allele parent*

Table 5. 2 - contingency and analysis for fertility restoration reaction as well as allelic classes observed in cross-2 (PHW- 1) population

Restoration reaction	Allelic classes for *Xwmc503*				2-value	P-value
	RR	AR	AA	NN	15.33	0.018*
Low	6	19	6	9		
Medium	6	11	5	3		
High	9	24	1	0		
	Allelic classes for *Xgwm296*					
Low	6	3	11	6	11.13	0.085
Medium	9	21	15	3		
High	3	11	8	3		

*significant at 0.05 probability level
R: restorer allele, A: sterility allele and N: nil allele
AA- Sterile homozygous parent

Table.6. Combined 2-analysis for marker-trait association for cross-1 and cross-2 (=0.05)

Cross	S. no.	Marker	Degrees of freedom	2-value	P-value	Result
CMS-BWL5203/R-6	1.	*Xwmc503*	(3-1)*(3-1)=4	9.84	0.043*	Associated
	2.	*Xgwm296*	(3-1)*(3-1)=4	6.88	0.142	Not associated
	3.	*Xwmc112*	-	-	-	Not amplified
PHW-1	1.	*Xwmc503*	(4-1)*(3-1)=6	15.33	0.018*	Associated
	2.	*Xgwm296*	(4-1)*(3-1)=6	11.13	0.085	Not associated
	3.	*Xwmc112*	-	-	-	Not amplified

*significant at 0.05 probability level

significant association with fertility restoration (=0.05) in case of cross-1 as well as in cross-2. In contrast to this, marker *Xwmc296* had P-value less than level of significance in both the crosses (Table 6). So, it did not show significant association with fertility restoration (=0.05). However,the third marker *Xwmc112* did not amplify in the any of the populations despite running the gradient PCR with wide range(55-61^0**C**). The presence of null allele was observed in 15% (*Xwmc503*) and 12% (*Xgwm296*) of the total F2 population exhibiting low to medium restoration. The genetic distance of these SSRs from *Rf8* (Table 1) could have led to the occurrence of null alleles in significant proportion of the population.

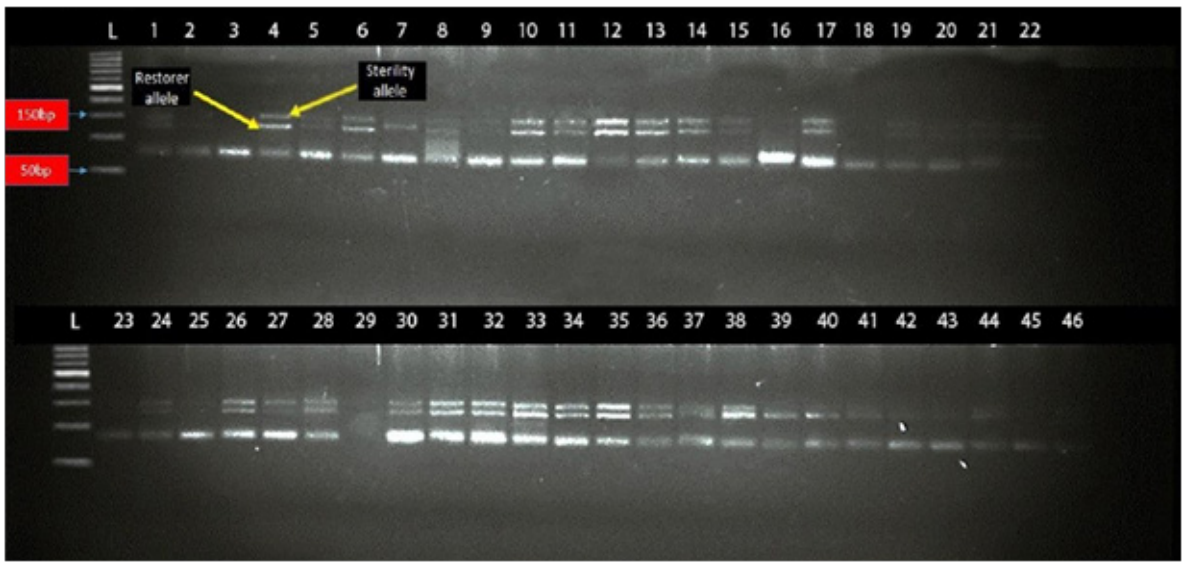

Fig 2. Amplification pattern showing 1-46 F_2 plants (cross-1) segregating for *Rf8* gene with marker *Xgwm296,* with 'L' being the standard DNA ladder (50 bp)

4. Conclusion

Thus, for *Triticum timopheevii* background, the marker *Xwmc503* linked with *Rf8* gene can prove to be useful for selecting plants possessing this very gene for fertility restoration to transfer it into elite cultivars. Then, those cultivars once incorporated with *Rf8* gene could positively be evaluated for other agronomic characteristics to further ensure their use as restorer parents in A X R programmes under wheat heterosis breeding. Restorer breeding can be sped up efficiently with MAS by analyzing the population against this robust marker in the early generations of material development. So, this closely linked *Xwmc503* marker could be useful in breeding programs such as marker assisted backcross breeding in order to enrich the male parental pool from the prospect of hybrid wheat.

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