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

Harmeet Singh Bakala1, Puja Srivastava, Vinod2, Ankita, Achla Sharma1, Gurvinder Singh Mavi1

1Department of Plant Breeding & Genetics, Punjab Agricultural University, Ludhiana-141004, India
2Division of Genetics, Indian Agricultural Research Institute, Pusa, New Delhi-110012, India

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 F2 populations 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 Rf8. 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 Rf8 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 heterosis in 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), thermophotosensitive 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 F₁ 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 (Rf) 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 (Virmanti 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 Xumc406 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 Rf8gene. 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-1 having 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 off-season research station, Keylong (Lahaul&Spiti, Himachal Pradesh) in May 2018. The low relative humidity over there promotes cleistogamy 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<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> 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 plants from 2 crosses. Tissue lyzer 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.

| S.no. | SSR Marker        | Marker efficacy (%) | Permissible errors (for 210 plants)* | Ta [0°C] | 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
(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 \( \leq 4 \) seeds per spike were grouped as sterile to the fertile ones having \( \geq 5 \) 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.

### Table 2

| Class               | Genotype          | Expected ratio | Observed no of plants | Expected no of plants | 2-value | P-value |
|---------------------|-------------------|----------------|-----------------------|-----------------------|---------|---------|
| Fertile (\( \geq 5 \) seeds/spike) | RaRbRbRb* RaRbRbRa RaRbrbRa RbRaRbrb | 2.79 63 | 9 51 | 4.78 | 0.028 |
| Sterile (0-4 seeds/spike) | Rbarbrb barbbrbRb Rbarbrbbrb Rbarbrbrb | 2.79 48 | 7 60 | 4.78 | 0.028 |
| 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 our study 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.

### 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 closest 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 are 10.12, 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 study was both the fertility restoration and Rf8 markers are independent variables otherwise association between both the characters under study (H1).

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 the combined \( \chi^2 \)-analysis of 2 linked SSR markers in table 6, it is clear that for \( X_{\text{wmc}503} \), the P-value is less than level of significance in both the crosses, rejecting the null hypothesis. Therefore, \( X_{\text{wmc}503} \) showed

### Table 4. \( \chi^2 \)-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 \( X_{\text{wmc}503} \) | \( \chi^2 \)-value | P-value |
|----------------------|------------------------------------------|------------------|---------|
|                      | RR | AR | NN |            |       |
| Low (\( \leq 5\%\)) | 7  | 14 | 14 | 9.84      | 0.043*|
| Medium (6-40%)       | 24 | 32 | 17 |           |        |
| High (\( \geq 40\%\)) | 4  | 1  | 0  |           |        |

| Allelic classes for \( X_{\text{gwm}296} \) | \( \chi^2 \)-value | P-value |
|--------------------------------------------|------------------|---------|
| Low (\( \leq 5\%\)) | 3 | 33 | 15 | 6.88 | 0.142 |
| Medium (6-40%) | 0 | 42 | 12 |     |       |
| High (\( \geq 40\%\)) | 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. \( \chi^2 \)-contingency and analysis for fertility restoration reaction as well as allelic classes observed in cross-2 (PHW-1) population

| Restoration reaction | Allelic classes for \( X_{\text{wmc}503} \) | \( \chi^2 \)-value | P-value |
|----------------------|------------------------------------------|------------------|---------|
|                      | RR | AR | AA | NN |            |       |
| Low                  | 6  | 19 | 6  | 6  | 15.33      | 0.018*|
| Medium               | 6  | 11 | 5  | 3  |           |        |
| High                 | 9  | 24 | 1  | 0  |           |        |

| Allelic classes for \( X_{\text{gwm}296} \) | \( \chi^2 \)-value | P-value |
|--------------------------------------------|------------------|---------|
| Low (\( \leq 5\%\)) | 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 \( \chi^2 \)-analysis for marker-trait association for cross-1 and cross-2 (\( \chi=0.05 \))

| Cross       | S. no. | Marker       | Degrees of freedom | \( \chi^2 \)-value | P-value | Result         |
|-------------|--------|--------------|--------------------|-------------------|---------|----------------|
| CMS-BWL 5203/R-6 | 1.     | \( X_{\text{wmc}503} \) | \( [3-1]*[3-1]=4 \) | 9.84              | 0.043* | Associated     |
|             | 2.     | \( X_{\text{gwm}296} \) | \( [3-1]*[3-1]=4 \) | 6.88              | 0.142  | Not associated |
|             | 3.     | \( X_{\text{wmc}112} \) | -                  | -                 | -      | Not amplified  |
| PHW-1       | 1.     | \( X_{\text{wmc}503} \) | \( [4-1]*[3-1]=6 \) | 15.33             | 0.018* | Associated     |
|             | 2.     | \( X_{\text{gwm}296} \) | \( [4-1]*[3-1]=6 \) | 11.13             | 0.085  | Not associated |
|             | 3.     | \( X_{\text{wmc}112} \) | -                  | -                 | -      | 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 any of the populations despite running the gradient PCR with wide range (55-61°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.

### 5. Acknowledgements

The authors acknowledge the funding received for this project through Consortia for Research Platform in Hybrid technology for higher productivity in selected field and horticultural crops-wheat, PC 2345

### References

1. Ali A, Vinod, SMS Tomar and S Chand. 2011. Genetics of fertility restoration and test for allelism of restorer genes in wheat (*Triticum aestivum* L.). *Indian Journal of Genetics* 71(3): 223-230.

2. Ahmed TA, H Tsujimoto and T. Sasakuma. 2001. QTL analysis of fertility-restoration against cytoplasmic male sterility in wheat. *Genes and Genetic Systems* 76: 33-38.

3. Bahl PN and SS Maan. 1973. Chromosomal location of fertility-restoring genes in six lines of common wheat. *Crop Science* 13: 317-320.

4. Boeven PHG, CFH Longin and T Würschum. 2016. A unified framework for hybrid breeding and the establishment of heterotic groups in wheat.
5. Du H, SS Maan and JJ Hammond. 1991. Genetic analyses of male fertility restoration in wheat. III. Effects of aneuploidy. Crop Science 31: 319-322.

6. Engledow FL and BP Pal. 1934. Investigations on yield in cereals I: VIII. Hybrid vigour in wheat. Journal of Agricultural Science 24: 390-409.

7. Freeman GF. 1919. Heredity of quantitative characters in wheat. Genetics 4: 1-9.

8. Geyer M, A Bund, T Albrecht, L Hartl and V Mohler. 2016a. Distribution of the fertility-restoring gene Rf3 in common and spelt wheat determined by an informative SNP marker. Molecular Breeding 36: 167.

9. Geyer M, A Bund, T Albrecht, L Hartl and V Mohler. 2016b. Improving fertility restoration and seed production efficiency in CMS hybrid wheat. 2nd HEZagrar PhD Symposium, Germany 2: 14-15.

10. Geyer M, T Albrecht, L Hartl and V Mohlar. 2018. Exploring the genetics of fertility restoration controlled by Rf1 in common wheat (Triticum aestivum L.) using high-density linkage maps. Molecular Genetics and Genomics 293: 451-462.

11. Hughes WG and JJ Bodden. 1977. Single gene restoration of cytoplasmic male sterility in wheat and its implications in the breeding of restorer line. Theoretical and Applied Genetics 50: 129-135.

12. Johnson VA, JW Schmidt and PJ Mattern. 1967. Hybrid wheat in the United States. Plant Foods for Human Nutrition 14: 193-211.

13. Johnson JW and FL Patterson. 1977. Interaction of genetic factors for fertility restoration in hybrid wheat. Crop Science 7: 695-699.

14. Kihara H. 1951. Substitution of nucleus and its effects on genome manifestation. Cytologia 16: 177-193.

15. Kojima T, H Tsubamoto and Y Ogihara. 1997. High-resolution RFLP mapping of fertility restoration (Rf3) gene against Triticum timopheevi cytoplasm located on chromosome 1BS of common wheat. Genes and Genetic Systems 72: 353-359.

16. Ma ZQ and M E Sorrells. 1995a. Genetic analysis of fertility restoration in wheat using restriction fragment length polymorphisms. Crop Science 35: 1137-1143.

17. Ma ZQ, YH Zhao and ME Sorrells. 1995b. Inheritance and chromosomal locations of male fertility restoring gene transferred from Aegilops umbellulata Zhuk. to Triticum aestivum L. Molecular Genetics and Genomics 247: 351-357.

18. Maan SS. 1992. A gene for embryo–endosperm compatibility and seed viability in alloplasmic Triticum turgidum. Genome 35: 772-779.

19. Maan SS, K A Lucken and JM Bravato. 1984. Genetic analysis of male fertility restoration in wheat I. Chromosome location of Rf genes. Crop Science 24: 17-20.

20. Muhleisen J, HP Piepho, HP Maurer, CF Longin and JC Reif (2014) Yield stability of hybrids versus lines in wheat, barley, and triticale. Theoretical and Applied Genetics 127: 309-316.

21. Murray HG and WF Thompson. 1980. Rapid isolation of high molecular weight DNA. Nucleic Acids Research 8: 4321-4325.

22. Pickett AA. 1993. Hybrid wheat - results and problems. Paul Parey Scientific Publishers, Germany, p. 259.

23. Pugsley AT and RN Oram. 1959. Genetic male sterility in wheat. Australian Plant Breeding and Genetics Newsletter no. 14.

24. R Core Team (2015) R: A Language and Environment for Statistical Computing (Vienna, Austria, 2015); http://R-project.org/.

25. Robertson LD and BC Curtis. 1967. Monosomic analysis of fertility restoration in common wheat (Triticum aestivum L.). Crop Science 7: 431-435.

26. Sage GCM. 1972. The inheritance of fertility restoration in male sterile wheat carrying cytoplasm derived from Triticum timopheevi. Theoretical and Applied Genetics 42: 223-243.

27. Sage GCM. 1976. Nucleo-cytoplasmic relationships in wheat. Advances in Agronomy 28: 267-300.

28. Schmidt JW and VA Johnson. 1963. Hybrid wheat. Advances in Agronomy 20: 199-232.

29. Schmidt JW, VA Johnson and SS Maan. 1962. Hybrid Wheat. Nebraska Agricultural Experimental Station Q 9: 9.

30. Schnable PS and RP Wise. 1998. The molecular basis of cytoplasmic male sterility and fertility restoration.
31. Shahinnia F, M Geyer, A Block, V Mohler and L Hartl. 2020. Identification of Rf9 and unravelling the genetic complexity for controlling fertility restoration in hybrid wheat. Preprint,DOI - 10.1101/2020.06.20.162644.

32. Shewry PR and SJ Hey. 2015. The contribution of wheat to human diet and health. Food and Energy Security 4(3):178-202.

33. Sinha P, SMS Tomar, Vinod, VK Singh and HS Balyan. 2013. Genetic analysis and molecular mapping of a new fertility restorer gene Rf8 for Triticum timopheevi cytoplasm in wheat (Triticum aestivum L.) using SSR markers. Genetica 141: 431-441.

34. Striff K, A Blouet and A Guckert. 1997. Hybrid wheat seed production potential using the chemical hybridizing agent SC2053. Plant Growth Regulators 21:103-108.

35. Tahir CM and K Tsunewaki. 1969. Monosomic analysis of Triticum spelta var. duhamelianum, a fertility restorer for T. timopheevi cytoplasm. Japanese Journal of Genetics 44: 1-9.

36. Tomar SMS, S Anabalgan and R Singh. 2004. Genetic analysis of male fertility restoration and red kernel colour in restorer lines in wheat (Triticum aestivum L.). Indian Journal of Genetics 64: 291-294.

37. Tomar SMS, P Sinha, A Ali, Vinod, B Singh and HS Balyan. 2009. Assessment of agro-morphological and molecular diversity among fertility restorer lines in wheat (Triticum aestivum L.). Indian Journal of Genetics 69: 183-190.

38. Tucker EJ, U Baumann, A Kouidri, R Suchecki, M Baes, M Garcia, T Okada, C Dong, Y Wu, A Sandhu, M Singh, P Langridge, P Wolters, M CAlbertsen, A M Cigan and R Whitford. 2017. Molecular identification of the wheat male fertility gene Ms1 and its prospects for hybrid breeding. Nature Communications 8: 869.

39. USDA. 2020.https://apps.fas.usda.gov/psdonline/circulars/production.pdf

40. Virmani SS and IB Edwards. 1983. Current status and future prospects for breeding hybrid rice and wheat. Advances in Agronomy 36: 145-214.

41. Whitford R, D Fleury, JC Reif, M Garcia, T Okada, V Korzun and P Langridge. 2013. Hybrid breeding in wheat: Technologies to improve hybrid wheat seed production. Journal of Exerimental Botany 64: 5411–5428.

42. Würschum T, G Liu, PHG Boeven, CFH Longin, V Mirdita, E Kazman, Y Zhao and JC Reif. 2018. Exploiting the Rht portfolio for hybrid wheat breeding. Theoretical and Applied Genetics 131: 1433-1442.

43. Wilson JA and WM Ross. 1962. Male sterility interaction of the Triticum aestivum nucleus and Triticum timopheevi cytoplasm. Wheat Info Serv (Kyoto University) 14: 29-30.

44. Zhang Y, L Luo, C Xu, Q Zhang and Y Xing. 2006. Quantitative trait loci for panicle size, heading date and plant height co-segregating in trait-performance derived near-isogenic lines of rice (Oryza sativa). Theoretical and Applied Genetics 113: 361-368.

45. Zhou W, LK Frederic, LD Leslie and S Wang. 2005 SSR markers associated with fertility restoration genes against Triticum timopheevii cytoplasm in Triticumaestivum. Euphytica 141: 33-40.