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Genetics of fertility restoration and test for allelism of restorer genes in wheat (*Triticum aestivum* L.)

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Abstract

Mode of inheritance of fertility restorer (*Rf*) gene(s) for T type (*Triticum timopheevi* Zhuk.) sterile cytoplasm in wheat (*Triticum aestivum* L.) was studied using fertility restorer lines EC368169 and T2003R. A-line 2041A was crossed with EC368169 while another male sterile line 2019A, with T2003R to develop F_2 and BC_1 (sterile line x F_1) populations. The F_1's of all the crosses had more than 60% pollen fertility and 48 to 68 seed set per main spike. Based on the observations recorded on pollen and floret fertility, the F_2 and BC_1 segregants were categorized into fertile (> 30% pollen fertility and > 5% seed set/spike) and sterile (< 30% pollen fertility and < 5% seed set/spike) classes. The segregation pattern in F_2 and BC_1 generations showed that both the restorer lines carried a single dominant gene for fertility restoration. However, the frequency distribution of plants in terms of pollen fertility and seed set in the F_2 indicated that fertility restoration was affected in complex way by modifiers. Test for allelism between four restorer lines were also conducted to find out the similarity between the genes carried by restorer lines namely, EC368169, T2003R, PWR4099 and PWR4101. The progenies from three way crosses, [2019A/F_1(PWR4101/PWR4099)], [2041A/F_1(PWR4101/T2003R)], [2041A/F_1(WR4099/T2003R)], 2019A/F_1(EC368169R/T2003R)] and [2338A/F_1(EC368169R/PWR4099)] did not segregate for male sterility indicating that at least one common fertility restorer gene is present in all the four restorer lines.

Key words : Cytoplasmic male sterility, fertility restorer genes, inheritance, test for allelism, bread wheat

Introduction

Exploitation of heterosis or hybrid vigour has been amply demonstrated in several cross pollinated crops, such as maize, pearl millete and sorghum etc. [1-3]. However, heterosis breeding is limited in case of self pollinated crops. With constant and concerted efforts commercial hybrids have been produced in rice, a strictly self pollinated crop. Considerable increase in grain yields in rice prompted that heterosis breeding is possible in other cereal crops such as wheat having similar floral biology (chasmogamous flowers), which does not promote natural out crossing. Change in pollination system of wheat has already been made to facilitate hybrid breeding programme [4, 5]. As the demands for wheat are likely to go up, the supply of wheat can be maintained only through the proportionate increase in production. Among several strategies of enhancing wheat production, development of hybrid wheat appears to be one of the important approaches to enhance the levels of productivity under marginal and optimum input environments. Since limits to yield ceiling are not known, hybrid technology may be used as a strategy to solve problems associated with low productivity. A large number of cytoplasmic male sterile (CMS) lines carrying cytoplasms from *Triticum timopheevi* Zhuk., *T. araraticum* Jakubz. and *Aegilops speltoides* Tausch, and *Ae. kotschyii* have been developed and characterized [6-8] and fertility restorer lines identified [9]. For successful exploitation of heterosis both stable cytoplasmic male sterile lines and genetically divergent fertility restorers are needed. The crucial and most important requirement for stable performance of hybrids is the restoration of complete fertility in F_1 across different environments. Therefore, the success of hybrid wheat depends on the identification and development of genetically diverse and agronomically superior fertility restorers because it not only recovers hybrid fertility but also determines hybrid

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vigor. The objective of the present study was to study the mode of inheritance and to find out the number and nature of gene(s) restoring fertility in stable CMS lines. A test for allelism was also conducted to determine whether fertility restoration in the four restorer lines is due to same or different genes. Information of similarity or dissimilarity of fertility restorer genes would facilitate pyramiding of more than one gene in desirable genetic background.

Material and methods

Plant material

The plant material comprised four fertility restorer lines *viz*., EC368169R originated from France, (pedigree not known), T2003R (HD69/NP839//S310//NP830), PWR 4101(CBHW-R CHN 89R 4294 OCHN S-2 BV97 = EC414148), PWR4099 (CBHW-R CHN QI RR925 OCHN S-4 BV97 = EC414149) and three cytoplasmic male sterile (CMS) lines, namely, 2041A, 2019A and 2338A which have the same *Triticum timopheevi* cytoplasm but different nuclear genomes.

Development of populations

To study the mode of inheritance, the cytoplasmic male sterile line, 2041A was crossed with EC368169R and 2019A was crossed with T2003R. Mode of inheritance of fertility restorer genes in PWR 4099 and PWR 4101 have been already studied in our laboratory, however, whether these restorers carry same or different gene(s) is not known. Hence to find out the commonality of fertility restorer (R) gene(s) five crosses were made between the restorer lines, PWR4101/PWR4099, PWR4101/T2003R, PWR4099/T2003R, EC368169R/T2003R and EC368169R/PWR4099. The crossed seeds were grown in summer nursery at IARI Regional Station, Wellington (Tamil Nadu) during *kharif* 2009. The F_1 plants from two crosses *viz*., 2041A/EC368169R and 2019A/T2003R were selfed to raise F_2 populations as well as backcrossed with respective CMS lines keeping F_1 plants as male parent to get BC_1 populations. The F_1s (R X R) were crossed with CMS lines, [2019A/F_1 (PWR4101/ PWR4099)], [2041A/F_1 (PWR4101/ T2003R)], [2041A/F_1(WR4099/T2003R)], 2019A/F_1(EC368169R/T2003R)] and [2338A/F_1(EC368169R/PWR4099)] to produce BC_1 populations through three way crosses. The F_2 and BC_1 generations and F_1s from three way crosses were grown in the net house at Division of Genetics, IARI, New Delhi, during *rabi* 2009-10. The parents and segregating generations were grown in rows 30 cm apart with seed to seed distance of 10cm. The F_1, F_2 and BC_1 plants were selfed by covering the spikes with butter paper bags to prevent any chance of out-crossing.

Studies on pollen fertility

Study on pollen fertility (%) was conducted by collecting spikes from F_1 and F_2 plants just prior to anthesis. The anthers of three spikelets taken randomly from lower, middle and top portion of the spike were smeared in a drop of 0.5% Iodine (I) solution prepared in 2% Potassium Iodide (KI) on a glass slide. The number of dark stained and round pollen grains were considered fertile while reddish-brown or yellowish-brown and unstained were categorized as sterile. The pollen grains were counted under an optical microscope in three microscopic fields and finally mean was taken. Pollen fertility in per cent was then calculated by dividing number of fertile pollen grains observed by total number of pollen grains in the microscopic field and multiplying by 100. Plants were classified into different fertility-sterility groups *viz*., 0 to 30 per cent pollen fertility as sterile and 31-100 per cent as fertile category as per the scale proposed by Chaudhury *et al*. [10] and supported by Govinda Raj and Virmani [11]. For classical genetic analysis the plants in partially fertile group were pooled with fertile group whereas partially sterile group was merged with fully sterile category.

Scoring of seed set per main spike

For recording observation on seed set, the main spike was covered with butter paper bag at the time of spike emergence prior to anthesis and the spikelet fertility was computed by counting the number of seeds set per spike at maturity. For genetic analysis plants with a seed set of less than 5% were considered as sterile while those with more than 5% seed set [12] were categorized as fertile group. Based on the observations recorded on seed set in F_2 and BC_1 populations plants were classified into two groups.

Results and discussion

The F_1s from the crosses, 2041A x EC368169R and 2019A x T2003R were completely fertile indicating that both the restorer lines restored complete fertility in the cytoplasmic male sterile lines involved in the crosses. The complete fertility of F_1 indicated that fertility restoration is dominant over male sterility. The restoration of fertility in F_1 hybrids was inferred from both seed set and pollen fertility. About 55 seeds (range 48-62) per main spike were produced in each F_1 plant in both the F_1s, which was comparable to maintainer (B) and restorer (R) lines, which produced an average of 53 and 64 seeds, respectively. Pollen stainability by

Iodine-potassium iodide (round shaped and dark stained pollen grains) under light microscope also confirmed full fertility of F_1 plants.

Segregation based on pollen fertility

The study on mode of inheritance of fertility restoration was carried out in F_2 populations derived from the crosses 2041A x EC368169R and 2019A x T2003R. All the plants in both the F_2 populations were analyzed for pollen fertility using Iodine- potassium iodide staining. Pollen fertility varied from 0-100 per-cent. Frequency distribution of F_2 plants based on pollen fertility with a class interval of ten is given in Table1 and Fig.1 to know the clear cut fertile and sterile groups. The perusal of Fig. 1 showed two peaks; one for completely sterile plants and the other for fertile. The variation in pollen fertility (complete sterility to full fertility) observed may be due to genetic background of F_2 segregants. In the cross, 2041A x EC368169R, a total of 195 F_2 plants were scored for pollen fertility. Out of these, 119 plants were fertile, 18 partially fertile, 13 partially sterile and 45 plants were completely sterile. The different groups of completely and partially fertile plants in F_2 population were merged to form a fertile category, while partial and completely sterile plants were considered as sterile group for the purpose of genetic analysis. The data of two distinct groups were subjected to Chi-square analysis. Thus, after merging into two groups, 137 plants were classified in to fertile (F) group, while 58 in sterile (S) category (Table 2). The observed frequency of fertile and sterile plants in the F_2 populations corresponded with the expected Mendelian segregation ratio of 3 (F): 1 (S), with a chi-square (χ^2) value of 2.34 (P value = 0.10-0.20). Similarly, in another F_2 generation derived from the cross, 2019A/T2003R, a total of 210 plants were studied. Out of these, 131 plants were fertile, 24 partially fertile and 24 partially sterile whereas 31 plants were completely sterile. As per the procedure followed in the other F_2 population, 155 plants were categorized as fertile, while 55 were classified as sterile. The observed frequency of fertile and sterile plants fitted well to expected ratio of 3(F) : 1 (S) with χ^2 value of 0.158 (P value = 0.50-0.70). These results indicated the presence of a single dominant gene controlling fertility restoration in EC368169R and T2003R lines respectively.

Table 1. Frequency of F_2 Segregants for pollen fertility in the crosses 2041A /EC368169 and 2019A/ T2003R

Percent pollen fertility (class)	Number of plants	
	2041A x EC368169R	2019A x T2003R
0	45	31
1 – 10	7	14
11 – 20	3	6
21 – 30	3	4
31 – 40	7	5
41 – 50	5	7
51 – 60	6	12
61 – 70	8	13
71 – 80	22	31
81 – 90	36	37
91 – 100	53	50
Total	195	210

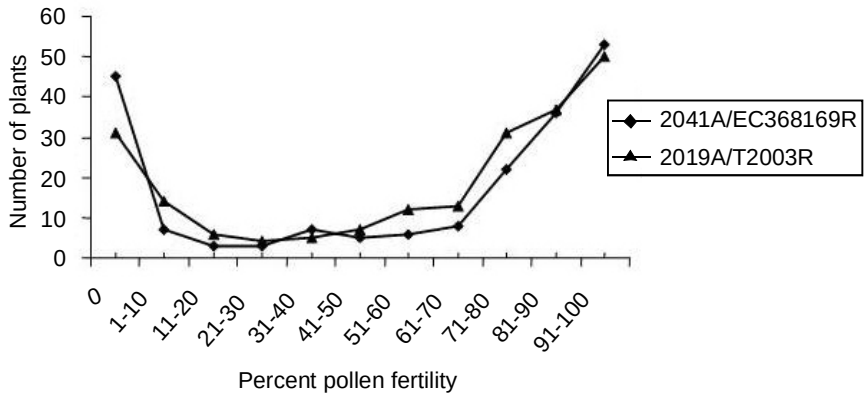

Fig. 1. Frequency distribution of pollen fertility in F_2 population in two crosses

However, the frequency distribution of the plants with respect to pollen fertility as given in Fig. 1 showed that the actual situation is much more complex, most probably due to segregation of the modifier genes in F_2. The distribution of F_2 plants showed probably a tendency towards partial dominance because of presence of modifiers and their variable degree of effects on phenotype. The Fig. 1 indicated inflection approximately at 10% and 60% pollen fertility. The modifiers

Table 2. Segregation of F_2 populations based on pollen fertility

Parent/cross	Generation	Fertile plants	Sterile plants	Total plants	Expected ratio (Fertile : sterile)	χ^2 value	P-value
2041A	P_1	0	11	11	-	-	-
EC368169R	P_2	12	0	12	-	-	-
(2041A/EC368169R)	F_2	137	58	195	3:1	2.34	0.10-0.20
2019A	P_1	0	13	13	-	-	-
T2003R	P_2	10	0	10	-	-	-
(2019A /T2003R)	F_2	155	55	210	3:1	0.158	0.50-0.70

influence largely the phenotypes with their heterozygous (*Rf rf*) genetic constitution but both in negative and positive directions. However, probably the F_1 (*Rf rf*) was not affected because of complementarity of fertility restoring genes and the modifiers.

Segregation based on spike seed set

The mode of inheritance for fertility restoration was also worked out on the basis of seed set per main spike in F_2 generation. Completely sterile plants are not expected to produce any seed whereas fully fertile plants should produce normal seed set in all the florets of a spike. It is expected that partial pollen fertility may result in reduced seed setting in a spike, which can not be considered as sterile. Based on the number of seeds produced per main spike of F_2 individuals derived from the crosses 2041A/EC368169R and 2019A/T2003R were classified into four categories as done for pollen fertility. The seed set per main spike ranged from zero to 82 and zero to 75 in both the F_2 populations, respectively. The seed set in respective B (maintainer) lines varied from 45 to 65. The seed set in F_2 segregants was plotted on a graph which like pollen fertility formed clear cut sterile and fertile groups (Fig. 2) rather than the normal distributions indicating that fertility restoration is not a polygenic trait. The present study considered the seed set per spike to classify the F_2 population for the purpose of genetic analysis. It would be better if the seed per spike was calculated as a percent of number of florets per spike. Out of 326 plants in the cross 2041A/EC368169R, 236 produced more than 31 seeds per spike and classed as fertile, and 90 plants formed <3 seeds and considered as sterile group. Thus, the observed frequency of fertile and sterile in the F_2 population corresponded with the expected frequency and fit well to Mendelian segregation ratio of 3 (F): 1 (S), with a chi-square (χ^2) value of 1.18 (P value = 0.30-0.20) at 1 degree of freedom (Table 3). Similarly in another F_2 generation derived from the cross 2019A X T2003R, 404 plants were scored for seed fertility. Based on the same criteria of seed set, 308 plants were classified as fertile and 96 plants were scored as sterile (Table 2). The observed frequency of fertile and sterile plants in the F_2 population corresponded with the expected frequency fitting well into Mendelian segregation ratio of 3 (F): 1 (S), with a chi-square (χ^2) value of 0.329 (P value = 0.70-0.50) at 1 degree of freedom. These results indicated the presence of a single dominant gene controlling fertility restoration in EC368169R and T2003R lines, respectively. The correspondence between pollen fertility and seed set could not be established as the data were recorded independently.

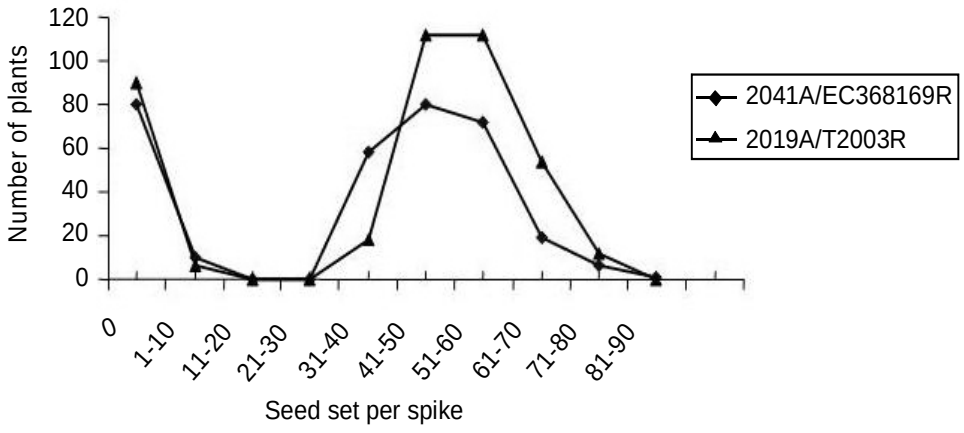

Fig. 2. Frequency distribution for seed set in F_2 population in two crosses

Studies on mode of

Table 3. Segregation of F_2 and BC_1 populations inferred on the basis of spikelet fertility

Parent/cross	Generation	Fertile plants	Sterile plants	Total plants	Expected ratio (Fertile : sterile)	χ^2 value	P-value
2041A	P_1	0	14	14	-	-	-
EC368169R	P_2	13	0	13	-	-	-
2041A/EC368169R	F_1	10	0	10	-	-	-
	F_2	236	90	326	3:1	1.18	0.30-0.20
2041A/F_1	BC_1	135	115	250	1:1	1.6	0.20
2019A	P_1	0	13	13	-	-	-
T2003R	P_2	12	0	12	-	-	-
2019A/T2003R	F_1	10	0	10	-	-	-
	F_2	308	96	404	3:1	0.329	0.70-0.50
2019A × F_1	BC_1	114	126	240	1:1	0.6	0.50-0.30

inheritance and determination of nuclear genes conditioning male fertility restoration in wheat conducted by Wilson [13] revealed that one major factor and some minor factors are necessary for full fertility restoration. Schmidt and Johnson [14] reported that two dominant genes are necessary for fertility restoration. Earlier, Maan [15] had also reported both dominant and recessive genes restoring fertility in wheat. Tomar *et al.* [9] also indicated that fertility restoration in wheat was governed by two independent dominant genes and the segregation pattern showed a semi-epistatic interaction, of these one has major effect. The range in seed set (> 5 % to 100 %) observed in F_2 generation comprising fully fertile and partially fertile plants correspond in appearance to continuous phenotypic variation governed by a single major gene which converts an otherwise qualitative character into quantitative one. It is therefore, assumed that some modifying genes have conspicuous effect on the phenotype interacting in different genetic backgrounds. These modifying genes seem to have cumulative small effect on seed set controlled by a major *Rf* gene. These modifier genes affecting fertility restoration may be dispersed through out the genome and if their number is not determinable, it is not possible to cull out the effect of individual modifiers. Ahmed *et al.* [16] detected some major and minor QTLs distributed on the chromosomes 2A, 4B and 6A in *T. timopheevi* CMS system and 1B and 2B in *Aegilops kotschyi* CMS system. Zhou *et al.* [17] observed that *Rf3* gene behave partially as well as completely dominant manner in conferring fertility restoration. Two minor QTLs conferring fertility restoration were also identified on chromosomes 5A and 7D in their study. Nonaka *et al.* [18] observed that one dose of *Rfv*1 gene was enough to restore complete fertility in *Ae. kotschyi* cytoplasm. However, Ikaguchi *et al* [19] stated that a single dose of *Rfv*1 was insufficient to restore a high level of fertility. Chen [20] reported a new model (A-line/R*-line//R-line) to produce hybrid wheat with complete fertility restoration. Classical studies conducted in rice also indicated that fertility restoration is controlled by a single gene as well as two dominant genes in WA cytoplasm by different fertility restorer lines [10, 11, 21]. Similarly, Fu *et al.* [22] clarified

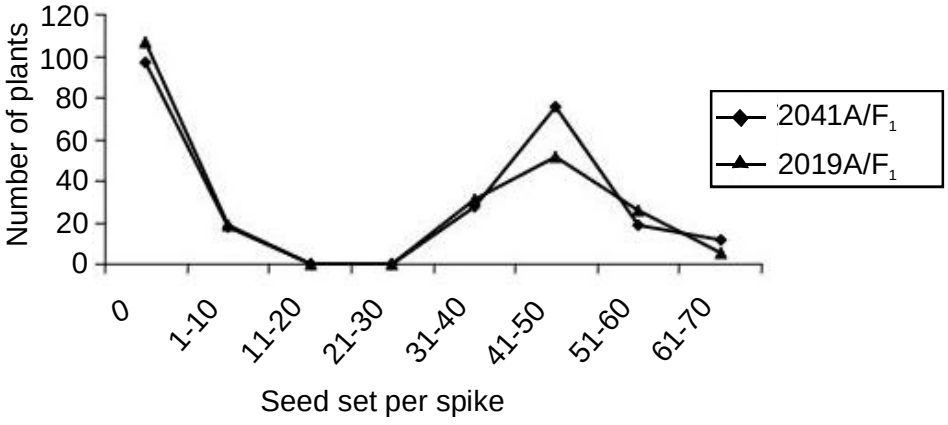

Fig. 3. Frequency distribution for seed set in BC_1 generation in two crosses

Table 4. Hypothetical model of inheritance of *Rf* gene

Sl. No.	Supposed genotype			Cross	Expected segregation fertile : sterile
	P_1	P_2	P_3		
1	$R_1R_1R_2R_2$	$R_1R_1r_2r_2$	$r_1r_1\ r_2r_2$	$r_1r_1\ r_2r_2$ x $F_1(R_1R_1R_2R_2$ x $R_1R_1r_2r_2$)	All fertile
2	R_1R_1	R_1R_1	r_1r_1	r_1r_1 x $F_1(R_1R_1$x R_1R_1)	All fertile
3	$R_1R_1\ r_2r_2$	$r_1r_1R_2R_2$	$r_1r_1\ r_2r_2$	$r_1r_1\ r_2r_2$ x $F_1(R_1R_1\ r_2r_2$x $r_1r_1R_2R_2$)	3(fertile):1(sterile)

Table 5. Observation of spikelet fertility of three way crosses

Cross	Plants		
	Fertile	Sterile	Total
2019A/F_1 (PWR4101/PWR4099)	401	2	403
2041A x F_1 (PWR4101/T2003R)	239	3	242
2041A/F_1 (PWR4099/T2003R)	223	4	227
2019[a]/F_1 (EC368169R/T2003R)	312	3	315
2338[a]/F_1 (EC368169R/PWR4099)	269	4	273

that one *Rf* gene in restorer lines T984 and H921 and two *Rf* genes in the restorer lines Milyang46 and H804 in rice controlled fertility restoration for ID-type CMS lines. This gene has been mapped on long arm of chromosome 10 in the fertility restorer line R68 [12]. Assuming that Rf_1Rf_1 and Rf_2Rf_2 are the dominant alleles of the two fertility restorer genes, the segregation pattern indicated that the plants homozygous for the recessive alleles ($rf_1rf_1\ rf_2rf_2$) of any one of the two genes but homozygous or heterozygous for the dominant alleles of the other gene ($Rf_1\_\ rf_2\ rf_2$ or $rf_1rf_1\ Rf_2\_$ or $Rf_1\_\ Rf_2\_$) will always show complete fertility in the segregating plants (Table 4). However, the results in present study indicated that single dominant gene is able to restore complete fertility in *T. timopheevi* cytoplasm.

Backcross generation

To test the validity of prediction of the gene hypothesis, the F_1 (*Rf*) was backcrossed to A line (*rf*). The BC_1 progenies were classified on the basis of seed set per spike. The BC_1 generation derived from the cross 2041A/F_1 (2041/EC368169R) produced approximately half of the progenies fertile and half sterile. Out of 250 plants from the test cross, 135 were fertile while 115 sterile. The observed frequency of BC_1 progenies fit well in the expected ratio of 1(fertile):1(sterile) with χ^2 value of 1.6 at P value 0.20, confirming the presence of a single dominant gene (Table 3) in fertility restorer line EC368169R. The minimum number of seed set in the fertile group was 31 (Fig. 3) that accounts for 75 per-cent seed set of the corresponding maintainer line. Similarly, the 240 progenies of the test cross 2019A/F_1 (2019/T2003R), consist of 114 fertile and 126 sterile. The observed phenotypic frequency appeared in a ratio approaching 1(fertile): 1(sterile) with the χ^2 value of 0.6 (P value = 0.50-0.30). The variation in seed set among the BC_1 progenies was also observed but the minimum number of seed set in the fertile group was 35 (Fig. 3) which accounts for 55 per-cent of the maintainer line HW 2019.

Test for allelism

Four lines identified as fertility restorers were crossed among themselves to produce five F_1 hybrids PWR4101/T2003R, PWR4099/T2003R, EC368169R/T2003R, PWR4101/PWR4099 and EC368169R/PWR4099. Hybrids thus obtained were crossed to different A lines. The progenies obtained from the three way crosses were observed for seed set per spike to determine the fertility. Out of 401 plants in the population derived from the cross 2019A x F_1 (PWR4101 x PWR4099), two plants did not set any seed and were considered sterile. Three plants were found sterile in a population of 242 plants from the cross 2041A/F_1 (PWR4101/T2003R). Just four plants, out of 227, were observed to be sterile from the cross 2041A/F_1 (PWR4099/T2003R). Similarly, three and four sterile plants were observed out of 315 and 273 individuals from the crosses 2019A/F_1 (EC368169R/T2003R) and 2338A/F_1 (EC368169R/PWR4099), respectively. The presence of very low frequency of sterile plants in each progeny derived from three way crosses (A-line/F_1) is presumably due to accumulation of modifiers having negative effect on fertility restoring gene in heterozygous condition. It is also possible that sterile plants are an admixture of A-lines (Table 5) and is not considered for (genetic analysis) test of allelism. These results implied that the fertility restorer genes present in all the four

fertility restorer lines are the same or at least one identical gene is present in all the four genotypes.

Tomar *et al*. [9] reported that the fertility restoration in PWR4101 and PWR4099 is controlled by one and two restorer gene(s) respectively. The progenies derived from three way crosses 2019A/F_1(PWR4101/PWR4099), 2338A/F_1(EC368169R/PWR4099) and 2041A/F_1(PWR4099/T2003R) did not segregate for sterility indicating that the gene present in PWR4101, EC368169R and T2003R is common with one of the gene present in PWR4099. Similarly, no segregation for sterility was observed in the progenies derived from the crosses 2041A/ F_1(PWR4101/T2003R) and 2019A/ F_1(EC368169R/T2003R) supports the view that only one dominant gene (*Rf*) restoring fertility is present in above mentioned lines and it is similar in its manifestation hence allelic to the gene present in PWR4101 and PWR4099. PWR4099 is an exotic fertility restorer line and is also diverse [23] hence, could have a different restorer allele. If we assume that the fertility restorer gene present in the two restorer lines crossed are the same then the F_1, when crossed with a CMS line will give all fertile progeny. If it is a different allele then the progeny will segregate. The hypothetical segregation of the different combinations of three way crosses is presented in Table 3. So far six *Rf* genes have been identified and located on specific chromosomes. The genes, *Rf1* on 1AS, *Rf2* on 7D, *Rf3* from *T. spelta duhamelianum* on 1B [17], *Rf4* on 6B, *Rf5* on 6D, Rf6 on 6AS and *Rf7* is located on short arm of 7B [24]. Yen et al [25] reported that *Rf4* and *Rf5* are located on 6D but Tahir and Tsunewaki [26] identified *Rf3* on short arm of chromosome1B. This kind of discrepancy existing on location of *Rf* genes could be resolved only by molecular mapping of these genes. Since the F_2 data of all the crosses studied indicated the presence of a single dominant gene in all the fertility restorer lines used in the study, only chromosomal location by cytological methods or molecular markers can further resolve the discrepancies. This study, keeping the complexity of segregation in view, may be worth carrying out in a more well designed manner in terms of parents included and mode of taking observations.

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