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"Selection 2427" carrying putative gametocidal gene(s)**

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Cytogenetic analysis and mapping of leaf rust resistance in *Aegilops speltoides*
Tausch derived bread wheat line “Selection 2427” carrying putative
gametocidal gene(s)

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Abstract

Leaf rust (*Puccinia triticina*) is a major biotic stress affecting wheat yields worldwide. Host plant resistance is the best method for controlling leaf rust. *Aegilops speltoides* is a good source of resistance against wheat rusts. So far five *Lr* genes, *Lr28*, *Lr35*, *Lr36*, *Lr47* and *Lr51* have been transferred from *Ae. speltoides* to bread wheat. In Selection2427, a bread wheat introgressed line with *Ae. speltoides* as donor parent, a dominant gene for leaf rust resistance was mapped to the long arm of chromosome 3B (*LrS2427*). None of the *Lr* genes introgressed from *Aegilops speltoides* has been mapped to chromosome 3B. Since none of the designated seedling leaf rust resistance genes have been located on chromosome 3B, *LrS2427* seems to be a novel gene. Sel.2427 showed a unique property typical of gametocidal genes that when crossed to other bread wheat cultivars the F₁ showed partial pollen sterility as well as poor seed setting whilst Sel.2427 shows reasonable male and female fertility. Accidental co-transfer of gametocidal genes with *LrS2427* may have occurred in Selection2427. Though *LrS2427* did not show any segregation distortion and assorted independently of putative gametocidal gene(s), its utilization will be difficult due to the selfish behavior of gametocidal genes.

Key words: wheat, leaf rust, *Lr* gene, *Aegilops speltoides*, gametocidal

Introduction

Wheat (*Triticum aestivum* L.) is a major cereal crop of the world with global production of 729 million tons from an area of 220 million hectares (FAOSTAT, 2014). Leaf or brown rust caused by *Puccinia triticina* Eriks is one of the most important fungal diseases which prevails in all the wheat growing regions. Leaf rust is more frequent and widespread in occurrence than stem or stripe rust (Roelfs *et al.* 1992; Marasas *et al.* 2004; Kolmer 2005; Bolton *et al.* 2008) and therefore causing greater total annual losses worldwide (Huerta-Espino *et al.* 2011). Though rust diseases can be controlled by application of fungicides, genetic resistance remains the most economical and environment friendly method (Kolmer 1996; Sharma 2003; Keller *et al.* 2008). Till date, 77 leaf rust resistance genes (*Lr*) have been designated in the catalogue of gene symbols for wheat (McIntosh *et al.* 2017). Almost half of them have been introgressed from closely or distantly related species and genera into common wheat (Naik *et al.* 2015). Most of the leaf rust resistance genes native to *T. aestivum* have become ineffective to the prevalent races of *P. triticina*. Virulence has evolved even against some of the alien genes such as *Lr9* (Nayar *et al.* 2003) *Lr19* (Bhardwaj *et al.* 2005), *Lr26* (Nayar *et al.* 1991) and *Lr28* (Bhardwaj *et al.* 2010) which has increased the vulnerability of wheat to leaf rust (Tomar *et al.* 2014). Evolution of new virulent races is a continuing phenomenon which necessitates the search and deployment of new and effective rust resistance genes. Wild relatives of wheat are reservoir of useful genes and several rust resistance genes have been introgressed from wild species into common wheat (Ellis *et al.* 2014). *Aegilops speltoides* Tausch (2n=2x=14, SS genome), a wild progenitor of wheat belonging to the secondary gene pool is a potential source of resistance to leaf and stem rusts (Tomar and Kochumadhavan 1996; Tomar and Menon 2001). So far, only five leaf rust resistance genes, *Lr28* (McIntosh 1982), *Lr35* (Kerber and Dyck 1990), *Lr36* (Dvorak and Knott

1990), *Lr47* (Dubcovsky *et al.* 1998) and *Lr51* (Dvorak 1977) have been transferred from *Ae. speltoides* into common wheat. At Indian Agricultural Research Institute, New Delhi, a genetic stock Selection2427 (Sel.2427) carrying leaf rust resistance was developed by a cross involving *Ae. speltoides*. In earlier studies, genetic analysis of leaf rust resistance in Sel.2427 revealed a single dominant gene (Dhakate 2002), however, Durgesh *et al.* (2011) observed sterility in F₁ plants when Chinese Spring monosomic series was crossed with Sel.2427. It is known that some of the *Aegilops spp.* particularly those with S or C genome carry gametocidal (*Gc*) genes that induce sterility in gametes not carrying *Gc* gene (s) when introduced in the wheat nuclear genome (Endo 1979; Tsujimoto and Tsunewaki 1984). It might be possible that Sel.2427 carries *Gc* gene (s) besides the leaf rust resistance gene. In this paper, we report the cytogenetic analysis and molecular mapping of leaf rust resistance in Sel.2427.

Materials & Methods

Plant material and leaf rust pathotypes

Sel.2427, derived from the cross *Triticum aestivum* cv. Sonalika /*Aegilops speltoides* acc. No. EC 331772) // Sonalika and two susceptible cultivars, Agra Local and NI5439 were used to study the mode of inheritance and mapping of leaf rust resistance gene. Sel.2427 was crossed with Agra Local and NI5439 to develop F₁ and F₂ generations.

The pure inoculum of twenty five races of *Puccinia triticina* including the two most virulent races, i.e. 104-2 (syn. 21R31) and 77-5 (syn. 121R63-1), were obtained from the Indian Institute of Wheat and Barley Research (IIWBR), Regional Station, Flowerdale, Shimla. The races were maintained and multiplied on susceptible cultivar Agra Local under greenhouse conditions at the Division of Genetics, IARI, New Delhi.

90 Testing for leaf rust resistance

91 Ten-day-old seedlings were inoculated by spraying with hand sprayer and incubated for
92 48 h in a humid glass chamber. After incubation, the seedlings were shifted to greenhouse
93 benches at temperatures ranging between 20 and 25 °C under ambient light and relative humidity
94 conditions. Later, the individual seedlings were scored for the leaf rust reaction (infection type)
95 at 12 days after inoculation using the 0–4 scale as described by Stakman *et al.* (1962).

96 Meiotic analysis in pollen mother cells (PMCs)

97 Cytological analysis of parents, F₁ and F₂ plants was carried out during meiotic
98 metaphase I of Pollen Mother Cells (PMCs). Anthers were collected from young spikes before
99 ear emergence at boot stage. One anther each from a floret was stained with 2 per cent
100 acetocarmine. If meiotic metaphase I stage was detected, remaining two anthers were fixed in 3:1
101 fixative (Carnoy's fluid: 3 parts absolute ethanol and 1 part glacial acetic acid). The fixed anthers
102 were hydrolyzed in 1N HCl at 60°C for 12 minutes. After draining 1N HCl, anthers were stained
103 in Feulgen for minimum of 30 minutes. Slides were prepared by squashing anthers in a drop of 2
104 per cent acetocarmine. Photos of chromosomal configurations were taken using Nikon
105 OPTIPHOT-2 compound microscope.

106 Pollen fertility and Seed set

107 Pollen fertility of parents, F₁ and F₂ plants was analyzed by staining pollen grains in
108 potassium iodide solution. Anthers were collected from three spikelets taken randomly from
109 lower, middle and top portion of the main spike and smeared on a glass slide in a solution
110 containing 0.5 per cent iodine prepared in 2 per cent potassium iodide. Pollen grains were
111 examined under light microscope in three different microscopic fields with 4X magnification.

Fertile pollen grains stained black and sterile ones appeared yellow or transparent. The pollen grains were categorized into two groups viz., fertile and sterile. The average number of seed set per spike was also determined for these plants to learn about female fertility.

Genomic DNA isolation

After screening for leaf rust resistance, the F₂ seedlings were transplanted in net house. Leaf samples were collected from 40 to 45-day-old plants and DNA was isolated by CTAB method (Murray and Thompson 1980). DNA samples were quantified by comparing with 100ng/200ng of lambda uncut DNA on 0.8% agarose gel and diluted to a final concentration of 20 - 25 ng/μl for PCR analysis.

SSR analysis

Parental polymorphism survey was conducted on parental lines Sel.2427, Agra Local and NI5439 with 741 SSR markers covering all the 21 chromosomes. The primer sequences of SSR markers used in this study were obtained from the GrainGenes website <http://www.graingenes.pw.usda.gov>. The SSR marker analyses were carried out in 10μl reaction volumes containing 4 mM Tris- HCl (pH 8.0), 20 mM KCl, 0.8 mM MgCl₂, 40 μM of each dNTP (MBI Fermentas, Germany), 1.0 unit Taq DNA polymerase (Bangalore Genei Pvt Ltd, India), 5 pM each of forward and reverse primers and 20 ng of genomic DNA. The polymerase chain reaction (PCR) was carried out in 96-well PCR plates with thermal seal in an Eppendorf thermal cycler (model Mastercycler pro S, Hamburg, Germany; www.eppendorf.com) with the following thermal profile: initial denaturation step of 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min (denaturation), 50–61 °C for 1 min (primer annealing) and 72 °C for 1 min (primer extension), with a final extension of 72 °C for 10 min. MetaPhor™ (Lonza) agarose gel

134 (3.5 %) was used to resolve the amplified PCR products in 1X TBE buffer and they were
135 visualized by ethidium bromide staining.

136 Bulk segregant analysis

137 Bulk segregant analysis (Michelmore *et al.* 1991) was employed to identify SSR
138 markers linked to the leaf rust resistance gene. Equal amount of DNA from 10 resistant and 10
139 susceptible F₂ plants was pooled to constitute the contrasting resistant and susceptible bulks,
140 respectively. The resistant and susceptible bulks along with parents were tested with
141 polymorphic SSR markers to identify putatively linked SSR markers.

142 Linkage analysis

143 The putative markers identified in BSA were used to genotype the entire F₂ population to
144 determine the number of recombinants produced by each marker. Linkage analysis was done
145 using the software MAPMAKER version 3.0 (Lander *et al.* 1987). The recombination
146 frequencies were converted to map distances in centimorgan (cM) using the option of Kosambi
147 function in the software (Kosambi 1943).

148 Statistical analysis

149 The segregation ratios in F₂ were subjected to Chi square (χ^2) analysis to test the
150 goodness of fit to the theoretically expected Mendelian segregation ratios using the formula $\chi^2 =$
151 $\sum (O - E)^2/E$, where O = observed number of individuals and E = expected number of
152 individuals.

153 Results

154 Screening for leaf rust resistance

Sel.2427 was resistant to all the 25 leaf rust races used in the study with infection type (IT) ranging from '0;' to '2⁺' against different races. In contrast, Agra Local was found susceptible (ITs '3' to '3⁺') to all the races. The second genotype used as susceptible parent in genetic analysis i.e. NI5439 was susceptible to most of the leaf rust races with IT ranging from 3= to 33+ except races 77 (ITs '1'), 104A (ITs '1⁺'), 104-3 (ITs '11⁺') and 108-1 (ITs '1') (Suppla).

Genetics of leaf rust resistance in Sel.2427

Genetic analysis of leaf rust resistance in Sel.2427 was undertaken in F₁ and F₂ generations derived from the crosses between Sel.2427 and the susceptible parents Agra Local and NI5439 against virulent races 104-2 and 77-5. Sel.2427 showed high degree of resistance against leaf rust races 104-2 and 77-5 with infection type (IT) '1' (Fig. 1a). Agra Local was susceptible against both leaf rust races (IT '3'). NI5439 showed susceptible reaction with IT '3' against 77-5 and '3' against 104-2 (Fig. 1b). The F₁ plants in all the crosses showed mesothetic resistant reaction with infection type 'X' (Fig. 1c). The F₂ populations segregated for leaf rust resistance showing resistant (Fig. 1d-g) as well as susceptible plants (Fig. 1h). All the six populations showed segregation with a goodness of fit to 3 resistant: 1 susceptible (Table: 1) indicating that Sel.2427 carries a single dominant leaf rust resistance gene and is hereafter abbreviated as *LrS2427*.

Cytological Analysis in Sel.2427, F₁ and F₂ plants

Cytological analysis of Sel.2427 during meiotic metaphase I revealed that most of the PMCs had normal meiotic configurations with 21 bivalents (Fig. 2a). Out of 151 PMCs analyzed from seven plants of Sel.2427, 119 PMCs showed 21 bivalents (Table: 2). However, in remaining PMCs unstable configurations like univalent, trivalent and quadrivalent were observed

besides presence of an isochromosome. One or two univalents were observed in 18 PMCs (Fig. 2b, d, e, f); quadrivalent in 13 PMCs (Fig. 2c, d); and a trivalent in four PMCs (Fig. 2f). Ten PMCs carried an isochromosome also. (Fig. 2d, e) (Table: 2). In Sel.2427, the number of bivalents ranged from 16 to 21 with an average of 20.66 ± 0.06 bivalents per PMC. Univalents, trivalent and quadrivalent were observed at low frequency with an average of 0.18 ± 0.04 (range 0-2), 0.03 ± 0.013 (range 0-1) and 0.09 ± 0.02 (range 0-1) per PMC, respectively. The number of isochromosomes per PMC varied from 0 to 1 with an average of 0.07 ± 0.02 (Table: 3). When Sel. 2427 was crossed to bread wheat lines Agra Local and NI5439, there was a drastic reduction in the frequency of PMCs with 21^{II}. Out of 61 PMCs analyzed in different F₁ (Sel. 2427 X Agra Local) plants, perfect 21 bivalents were observed in only five PMCs while 52 PMCs had one to eight univalent(s) (Fig. 3a-f). One quadrivalent was observed in four PMCs (Fig. 3f) and a trivalent in three PMCs (Fig. 3d, e). An isochromosome was also found in four PMCs (Table: 2). Average number of bivalents observed per PMC was 19.61 ± 0.12 (range 17–21), while the number of univalents ranged from 0 to 8 with an average of 2.31 ± 0.22 . The number of trivalents per PMC varied from 0 to 1 with an average of 0.05 ± 0.03 . Similarly, quadrivalents were observed at a low frequency of 0 to 1 with an average of 0.07 ± 0.03 . Isochromosomes ranged from 0 to 1 with an average of 0.07 ± 0.03 (Table: 3). Out of 85 PMCs analyzed in different plants of reciprocal F₁ (Agra Local X Sel. 2427), only nine PMCs displayed 21 bivalents; 71 PMCs showed one to eight univalent(s) (Fig. 3a-f); five PMCs had one quadrivalent (Fig. 3f) and two PMCs one trivalent (Fig. 3d, e). Five PMCs also showed presence of one isochromosome (Table: 2). The number of bivalents per PMC ranged from 17 to 21 with an average of 19.68 ± 0.09 while univalents varied from 0 to 8 with an average of 2.22 ± 0.17 . The frequency of trivalent and

quadrivalent was low with an average of 0.02 ± 0.02 and 0.07 ± 0.03 , respectively. Isochromosomes (0 to 1) were observed with an average of 0.06 ± 0.03 per PMC (Table: 3).

Cytological analysis was also carried out in nine randomly selected F_2 plants. The cytological behavior of three F_2 plants was similar to Sel.2427 in the sense that the number of PMCs with 21^{II} (Fig. 4a) was high with occasional appearance of univalents, trivalents and isochromosomes (Table: 2). In four plants, the number of PMCs with 21^{II} was low with higher frequency of PMCs with 1-8 univalents (Fig. 4c, d, f). Other cytological configurations like isochromosome (Fig. 4d) and quadrivalent (Fig. 4e) were also observed. In one F_2 plant, a monosomic chromosome number with $2n=41$ was observed, indicating loss of a chromosome. In all the 19 PMCs of this plant which were analysed, the configuration of $20^{II} + 1^I$ was consistent (Fig. 4b). In case of the last F_2 plant, not a single PMC with 21^{II} was observed. It showed a higher frequency of PMCs with univalents ranging from 2 to 6 and occasional appearance of a quadrivalent (Fig. 4e). Overall, in the F_2 generation the number of bivalents per PMC ranged from 16 to 21 with an average of 20.18 ± 0.08 while univalents ranged from 0 to 6 with an average of 1.27 ± 0.13 . Trivalent and quadrivalent varied from 0 to 1 with an average of 0.01 ± 0.01 and 0.04 ± 0.02 , respectively. The number of isochromosomes ranged from 0 to 1 with an average of 0.04 ± 0.02 (Table: 3).

Study of pollen fertility (%) and average seed set per spike

Sel.2427 exhibited pollen fertility ranging from 79.61 per cent to 92.57 per cent with a mean value of 87.96 per cent. The seed set per spike ranged from 22.00 to 31.56 with a mean value of 26.86 (Table: 4). When Sel.2427 was crossed to bread wheat cultivars Agra Local and NI5439, there was further drop in pollen fertility (Supplb) and seed set. Pollen fertility of F_1

plants showed wide variation as compared to Sel.2427, ranging from 26.20 per cent to 73.46 per cent with a mean value of 51.48 per cent (Table: 4). Average seed set per spike was also low ranging from 2.00 to 9.92 with a mean value of 6.30 (Table: 4). Even though maximum pollen fertility of 73.46 per cent was observed it did not correspond to a higher seed set. The F_1 plants showed high levels of sterility irrespective of the maternal or paternal origin of Sel.2427, thus, ruling out reciprocal differences. Plants of two F_2 populations (Agra Local/Sel.2427 and NI 5439/Sel.2427) also showed wide variation in pollen fertility and seed set per spike (Supplc). Pollen fertility ranged from 3.38 per cent to 94.01 per cent in F_2 derived from the cross NI5439 X Sel. 2427 while in other F_2 population (Agra Local X Sel. 2427), it ranged from 3.03 per cent to 94.33 per cent. The seed set per spike ranged from 0 to 38 and 0 to 39, respectively in the two F_2 populations. In F_1 plants the minimum pollen fertility and seed set per spike observed was 26.20 per cent and 2.00, respectively. In F_2 population plants were observed with pollen fertility less than 10 per cent and seed set per spike as low as zero. Conversely, few plants with better pollen fertility (max 94.33 per cent) and seed set per spike (max 39) than Sel.2427 were also observed. Frequency distribution of both traits was skewed (Fig. 5) indicating involvement of major gene(s) in controlling sterility.

Molecular mapping of leaf rust resistance gene using microsatellite markers

Parental polymorphism survey was conducted with 741 microsatellite markers and out of these, 189 were polymorphic between Sel.2427 and Agra Local. Of these 189 polymorphic microsatellite markers, only three markers located on long arm of 3B chromosome namely *Xwmc527*, *Xwmc632* and *Xgpw4044* were found to be putatively associated with resistance in bulked segregant analysis (Fig.6). Two other microsatellite markers namely *Xgwm340* and *Xgwm547* which were polymorphic between parents were also utilized in genotyping F_2

populations. Microsatellite genotyping was done in reciprocal F_2 (Agra Local/Sel.2427 and Sel.2427/Agra Local) populations which were screened for resistance against leaf rust race 104-2. Joint segregation analysis and test of linkage was done to confirm the results. Out of the five markers, *Xwmc527*, *Xgwm547*, *Xwmc632* and *Xgwm340* behaved as co-dominant markers while *Xgpw4044* segregated as dominant marker in coupling phase. In both F_2 populations, all the co-dominant markers segregated in expected ratio of 1:2:1 except *Xwmc632*. The dominant marker *Xgpw4044* segregated in 3:1 ratio. In both the F_2 populations, joint segregation analysis between co-dominant markers *Xwmc527*, *Xgwm547*, *Xwmc632* and *Xgwm340* and leaf rust resistance gene revealed that the ratio of segregation deviated significantly from expected ratio of 3 (R-/MM): 6 (R-/Mm): 3 (R-/mm): 1 (rr/MM): 2 (rr/Mm): 1 (rr/mm). The dominant marker *Xgpw4044* was also found to be co-segregating with leaf rust resistance gene and the ratio deviated significantly from 9 (R-/M-): 3 (R-/mm): 3 (rr/M-): 1 (rr/mm) (Table: 5).

Linkage map construction

The co-segregation analysis for individual SSR marker genotype and disease reaction phenotype of 152 individual F_2 plants derived from the cross Agra Local X Sel.2427 was done using mapmaker version 3.0 software (LOD threshold 3.0; mapping function Kosambi). Based on the linkage analysis the following best order was chosen: *Xwmc527*, *Xgwm547*, *Xwmc632*, *LrS2427*, *Xgpw4044* and *Xgwm340*. The position of linked SSR markers in relation to the leaf rust resistance gene, abbreviated as *LrS2427* is shown in Fig. 7(a). *LrS2427* is flanked by *Xwmc632* and *Xgpw4044*. The closest marker linked to *LrS2427* is *Xgpw4044* (24.1 cM; LOD: 21.34). In the reciprocal F_2 population involving 123 plants, similar results were obtained (Fig. 7b). The marker *Xgpw4044* is located at a map distance 19.7 cM (LOD=22.58).

Discussion

Sel.2427 was found to carry a dominant leaf rust resistance gene *LrS2427* which showed effectiveness against all the 25 leaf rust races, including the predominant virulent races 77-5 and 104-2 used in this study. *LrS2427* was mapped to the long arm of 3B chromosome. Out of 77 *Lr* genes catalogued, only one gene *Lr77* has been reported on long arm of 3B chromosome (McIntosh *et al.* 2017). Unlike the leaf rust resistance gene in Sel.2427 (*LrS2427*) which is a seedling resistance gene, *Lr77* is an adult plant resistance (APR) gene. Seedling resistance genes in wheat are effective at all the growth stages of plant whereas APR genes show susceptible reaction at seedling stage (Singh *et al.* 2001). Thus, the nature of leaf rust resistance gene *LrS2427* is different from *Lr77* indicating that it is a diverse gene. The chromosomal location as well as the nature of *LrS2427* suggests that it is a diverse leaf rust resistance gene, which may be useful in resistance breeding programs. Recently leaf rust resistance conditioned by a single incompletely dominant gene mapping to 3BL has also been reported in a *T. timopheevii*-derived bread wheat line (Singh *et al.* 2017). Molecular mapping is very useful in determining the identity of new genes (Gireesh *et al.* 2014, 2015). The closest marker *Xgpw4044* is mapped at 24.1 or 19.7 cM from the leaf rust resistance gene in the two mapping populations. Even though SSR marker analysis has suggested the novelty of the gene, the linked markers show 20% or higher recombination frequency, hence not useful in marker assisted selection for resistance gene. Cytological and fertility/sterility analysis showed that Sel.2427 also carries some undesirable genetic elements which are responsible for meiotic instability as well as partial fertility of plants when crossed with other bread wheat lines. This is the unique property of gametocidal genes. Due to the association of such factors validation of mapping in F₂ could not be carried out in advanced generations.

Endo (1979; 1990; 2007) described gamete killing genes in *Ae. cylindrica* and named them gametocidal genes (*Gc*) and the chromosomes carrying these genes as gametocidal chromosomes. Gametocidal genes have been reported on chromosome 2C of *Ae. cylindrica* (Endo 1979), chromosome 3C of *Ae. triuncialis* and *Ae. caudata* (Endo and Tsunewaki 1975), chromosome 2S or 6S of *Ae. speltoides* (Tsujiimoto and Tsunewaki 1984; 1988; Kota and Dvorak 1988) and chromosome 4M of *Ae. geniculata* (Kynast *et al.* 2000). Sel.2427 is cytologically unstable and on crossing with *T. aestivum* cultivars exhibit high frequency of meiotic configurations like univalents, multivalents, isochromosomes and lagging chromosomes. Chinese Spring disomic alien addition lines carrying gametocidal chromosome 2C when crossed with Chinese Spring-*Lophopyrum elongatum* 6E and *Elytriga* disomic addition lines exhibited a high frequency of univalents and a moderate frequency of multivalents (Zhao *et al.* 2013; Liu *et al.* 2007). Similarly, gametocidal chromosome 2S was also reported to induce chromosome breaks in *Triticum aestivum* cultivar *Ningnong* and 5R/5A wheat-rye substitution lines (Chen *et al.* 2008). Presence of multivalents and isochromosomes suggest the breakage of chromosomes and gametocidal genes are known to cause sterility in gametes lacking *Gc* gene(s) by causing fragmentation of chromosomes (Finch *et al.* 1984; Nasuda *et al.* 1998).

In a plant heterozygous for *Gc* gene (s), the gametes lacking *Gc* gene become abortive (Tsujiimoto 2005) leading to partial sterility. In the F₂ population, segregation of *Gc* gene(s) leads to wide variation in fertility which is clearly noticeable in Sel.2427. Tsujiimoto and Tsunewaki (1984; 1988) identified two *Gc* genes *Gc1a* and *Gc1b* from *Ae. speltoides* which have been transferred independently to chromosome 2B of common wheat. The near isogenic line of Chinese Spring with *Gc1a*, being homozygous for *Gc1a* was morphologically the same as Chinese Spring without *Gc1a*, except for a slight reduction of fertility from 99.3 to 96.5 per cent (Tsujiimoto and Tsunewaki 1983). When it was crossed to Chinese Spring, shriveled seeds that germinate at low frequency were

314 obtained (Tsujiimoto and Tsunewaki 1988). Gametocidal genes in different species exhibit varying
315 levels of strength. For instance, gametocidal genes from the Sitopsis section viz., *Ae. longissima*, *Ae.*
316 *sharonensis* and *Ae. speltooides* have strong gametocidal action (Deven, 2007) while *Ae. triuncialis* and
317 *Ae. cylindrica* carry weak gametocidal genes (Endo 1990). If a single gene is responsible for gametocidal
318 activity in Sel.2427, heterozygosity will lead to death of almost half of the gametes lacking 'Gc' in male
319 and female counterparts due to strong gametocidal action. In that case when F₁ is selfed it will result only
320 in plants which are homozygous for Gc gene and the variation of pollen fertility and seed set per spike in
321 F₂ population would have been nominal. But here the F₂ population exhibits wide variation in both
322 traits indicating involvement of more than one gene. The skewed frequency distribution in F₂
323 population implies that the concerned gene(s) have major effects (Figure 5).

324 Nevertheless, the fact that leaf rust resistance gene in Sel.2427 segregated in normal 3:1
325 ratio suggests that it is independently assorting and not linked to the putative Gc gene(s). Multiple
326 introgressions might have occurred from *Ae. speltooides* in Sel.2427 which can only be visualized by
327 genomic in situ hybridization techniques. Since gametocidal gene(s) ensure their preferential
328 transmission, utilization of rust resistance in Sel.2427 will be hampered. The strong gametocidal
329 activity in Sel.2427 necessitates rigorous measures like mutagenesis or one or more rounds of
330 induced homoeologous pairing to get rid of the undesirable gene(s). EMS mutagenesis was used to
331 produce a knock-out mutation of the Gc2 gene in wheat-*Ae. sharonensis* translocation
332 chromosome (T4B-4Ssh#1) which restored spike fertility (Friebe *et al.* 2003). Allosyndetic pairing
333 induction was used in an attempt to remove the Gc gene(s) from *Ae. speltooides* introgression lines having
334 rust resistance genes (introgressions S24 and S13 located on chr#3A) and putative primary recombinants
335 with improved fertility and plant type were obtained (Marais *et al.* 2010). These approaches can enable
336 the utilization of novel Lr gene in Sel.2427 for resistance gene deployment.

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Captions for figures

Fig. 1 Test for seedling resistance in F₂ population against leaf rust race 104-2. Disease reaction in (a) Sel.2427 showing ;1, (b) Agra Local showing 3, (c) F₁ (AL X S2427) showing X (d) to (h) Disease reaction observed in F₂ plants showing resistance (d-g) and susceptibility (h).

Fig. 2 Meiotic configurations observed at metaphase I stage in Sel.2427. PMCs showing (a) 21^{II} (b) 20^{II} + 2^I (c) 19^{II} + 1^{IV} (d) 18^{II} + 1^{IV} + 1^I + 1^{iso} (e) 20^{II} + 1^I + 1^{iso} (f) 19^{II} + 1^{III} + 1^I [Note: Arrows denote configurations like univalents (U), trivalent (T), quadrivalent (Q) and Isochromosome (I)]

Fig. 3 Meiotic configurations in PMCs of F_1 plants. PMCs showing configurations: (a) $20^{II} + 2^I$, (b) $19^{II} + 4^I$, (c) $18^{II} + 6^I$, (d) $18^{II} + 1^{III} + 3^I$ (e) $17^{II} + 1^{III} + 5^I$ (f) $17^{II} + 1^{IV} + 4^I$ [Note: Arrows denote configurations like univalents (U), trivalent (T), quadrivalent (Q) and Isochromosome (I)]

Fig. 4 Meiotic configurations in the PMCs of various F_2 plants. PMCs showing different configurations like (a) 21^{II} (b) $20^{II} + 1^I$ (c) $20^{II} + 2^I$ (d) $20^{II} + 1^I + 1^{iso}$ (e) $19^{II} + 1^{IV}$ (f) $18^{II} + 3^I + 1^{III}$ [Note: Arrows denote configurations like univalents (U), trivalent (T), quadrivalent (Q) and Isochromosome (I)]

Fig. 5 Frequency distribution for Average pollen fertility (%) and Average seed set per spike in F_2 populations of crosses (a) & (c) NI5439 X Sel.2427 (*rabi* 2013-14) and (b) & (d) Agra Local X Sel.2427 (*rabi* 2014-15).

Fig. 6 Result of Bulk Segregant Analysis (BSA). Three microsatellite markers namely *Xwmc527*, *Xwmc632* and *Xgpcw4044* were found to be polymorphic between Resistant and Susceptible bulks comprising of ten resistant and ten susceptible plants, respectively.

Fig. 7 Linkage map constructed for leaf rust resistance gene in (a) F_2 (Agra Local X Sel.2427) population and (b) F_2 (Sel.2427 X Agra Local) using five microsatellite markers. (Note: In parenthesis the LOD score is given)

Supplb Comparison of pollen fertility in (a) Agra Local (b) Selection 2427 (c) F_1 (Selection 2427 X Agra Local) and (d) F_1 (Agra Local X Selection 2427)

Supplc Variability for Average pollen fertility (%) and Average seed set per spike in Sel.2427 and F_1 and F_2 generations.

Table 1: Segregation of leaf rust resistance in F₂ population derived from crosses of Sel.2427 with susceptible parents Agra Local and NI 5439

Cross	Race	No. of F ₁ seedlings			No. of F ₂ seedlings			χ^2 (3:1)	P value
		Resistant	Susceptible	Total	Resistant	Susceptible	Total		
Agra Local X Sel.2427	104-2	5	0	5	118	35	153	0.368	0.544
Sel.2427 X Agra Local	104-2	5	0	5	130	31	161	2.835	0.092
NI 5439 X Sel.2427	104-2	5	0	5	51	22	73	1.028	0.311
Homogeneity χ^2					299	88	387	3.176	0.204
Agra Local X Sel.2427	77-5	5	0	5	65	28	93	1.294	0.255
Sel.2427 X Agra Local	77-5	5	0	5	58	22	80	0.267	0.605
Sel.2427 X NI 5439	77-5	5	0	5	79	33	112	1.190	0.275
Homogeneity χ^2					202	83	285	0.167	0.919

Table 2: Frequency of different configurations in Selection 2427, F₁ (Sel.2427 X Agra Local), reciprocal F₁ (Agra Local X Sel.2427) and F₂ (NI 5439 X Sel.2427)

Pl. No.		No. of PMCs observed	No. of PMCs with 21 ^{II}	No. of PMCs other than 21 ^{II}	Configurations involving only bivalents and univalents	Other configurations
Selection 2427	1	28	21	7	20 ^{II} +2 ^I (2)	20 ^{II} +1 ^I +1 ^{iso} (2), 19 ^{II} +1 ^{IV} (2), 19 ^{II} +1 ^{III} +1 ^I (1)
	2	17	12	5	-	20 ^{II} +1 ^I +1 ^{iso} (2), 20 ^{II} +1 ^{iso} (1), 19 ^{II} +1 ^{IV} (2)
	3	24	18	6	20 ^{II} +2 ^I (2)	19 ^{II} +1 ^{IV} (3), 19 ^{II} +1 ^{III} +1 ^I (1)
	4	22	18	4	-	20 ^{II} +1 ^I +1 ^{iso} (1), 19 ^{II} +1 ^{IV} (2), 19 ^{II} +1 ^{III} +1 ^I (1)
	5	16	16	0	-	-
	6	20	18	2	-	20 ^{II} +1 ^I +1 ^{iso} (1), 19 ^{II} +1 ^{IV} (1)
	7	24	16	8	20 ^{II} +2 ^I (3)	20 ^{II} +1 ^I +1 ^{iso} (2), 19 ^{II} +1 ^{IV} (2), 16 ^{II} +2 ^I +1 ^{IV} +1 ^{III} +1 ^{iso} (1)
	Total	151	119	32	-	-
F ₁	1	20	2	18	20 ^{II} +2 ^I (13), 17 ^{II} +8 ^I (1)	20 ^{II} +1 ^I +1 ^{iso} (3), 18 ^{II} +3 ^I +1 ^{III} (1)
	2	15	2	13	20 ^{II} +2 ^I (7), 19 ^{II} +4 ^I (3)	19 ^{II} +1 ^{IV} (3)
	3	12	1	11	20 ^{II} +2 ^I (7), 19 ^{II} +4 ^I (3)	19 ^{II} +1 ^I +1 ^{III} (1)
	4	14	0	14	20 ^{II} +2 ^I (6), 18 ^{II} +6 ^I (5)	20 ^{II} +1 ^I +1 ^{iso} (1), 17 ^{II} +5 ^I +1 ^{III} (1), 19 ^{II} +1 ^{IV} (1)
	Total	61	5	56	-	-
F ₁ (Reciprocal)	1	17	3	14	20 ^{II} +2 ^I (2), 19 ^{II} +4 ^I (5), 18 ^{II} +6 ^I (1), 17 ^{II} +8 ^I (1)	19 ^{II} +3 ^I +1 ^{iso} (3), 19 ^{II} +1 ^{IV} (2)
	2	30	2	28	20 ^{II} +2 ^I (21), 19 ^{II} +4 ^I (4), 18 ^{II} +6 ^I (1)	19+1 ^{IV} (2)
	3	15	2	13	20 ^{II} +2 ^I (11)	19 ^{II} +1 ^{IV} (1), 18 ^{II} +1 ^{IV} +2 ^I (1)
	4	23	2	21	20 ^{II} +2 ^I (12), 19 ^{II} +4 ^I (4), 17 ^{II} +8 ^I (1)	19 ^{II} +1 ^I +1 ^{III} (1), 18 ^{II} +3 ^I +1 ^{III} (1), 20 ^{II} +1 ^I +1 ^{iso} (2)
	Total	85	9	76	-	-
F ₂ (NI 5439 X Sel. 2427)	1	21	18	3	20 ^{II} +2 ^I (1)	19 ^{II} +1 ^{III} +1 ^{iso} (1), 20 ^{II} +1 ^I +1 ^{iso} (1)
	2	16	14	2	20+2 ^I (2)	-
	3	25	20	5	20+2 ^I (5)	-
	4	13	2	11	20 ^{II} +2 ^I (5), 19 ^{II} +4 ^I (3), 18 ^{II} +6 ^I (2)	19 ^{II} +1 ^{IV} (1)
	5	15	3	12	20 ^{II} +2 ^I (8), 19 ^{II} +4 ^I (4)	-
	6	12	2	10	20 ^{II} +2 ^I (5), 19 ^{II} +4 ^I (2), 18 ^{II} +6 ^I (1)	19 ^{II} +3 ^I +1 ^{iso} (1), 20 ^{II} +1 ^I +1 ^{iso} (1)
	7	9	2	7	20 ^{II} +2 ^I (3), 19 ^{II} +4 ^I (2)	20 ^{II} +1 ^I +1 ^{iso} (1), 19 ^{II} +3 ^I +1 ^{iso} (1)
	8	19	0	19	20 ^{II} +1 ^I (19)	-
	9	12	0	12	20 ^{II} +2 ^I (4), 19 ^{II} +4 ^I (1), 18 ^{II} +6 ^I (2)	19 ^{II} +1 ^{IV} (3), 18 ^{II} +1 ^{IV} +2 ^I (1), 16 ^{II} +1 ^{IV} +6 ^I (1)
	Total	142	61	81	-	-

Note: Number of PMCs showing different configurations is given in parenthesis

#I-Univalent; IV-Quadrivalent; III- Trivalent; Iso- Isochromosomes

* {} No. of univalents observed

Table 3: Metaphase I Chromosome association in Selection 2427, F₁ (Sel.2427 X Agra Local), reciprocal F₁ (Agra Local X Sel.2427) and F₂ (NI 5439 X Sel.2427)

Generation	Chr No.	No. of PMCs observed	Metaphase I Chromosome association				
			Univalent	Bivalent	Trivalent	Quadrivalent	Isochromosome
Selection 2427	42	151	0.18±0.04 (0-2)	20.66±0.06 (16-21)	0.03±0.013 (0-1)	0.09±0.02 (0-1)	0.07±0.02 (0-1)
F ₁ (Sel.2427 X Agra Local)	42	61	2.31±0.22 (0-8)	19.61±0.12 (17-21)	0.05±0.03 (0-1)	0.07±0.03 (0-1)	0.07±0.03 (0-1)
F ₁ (Agra Local X Sel.2427)	42	85	2.22±0.17 (0-8)	19.68±0.09 (17-21)	0.02±0.02 (0-1)	0.07±0.03 (0-1)	0.06±0.03 (0-1)
F ₂ (NI 5439 X Sel. 2427)	42 41	142	1.27±0.13 (0-6)	20.18±0.08 (16-21)	0.01±0.01 (0-1)	0.04±0.02 (0-1)	0.04±0.02 (0-1)

Table 4: Average Pollen fertility (%) and Average Seed set per spike in different plants of Sel.2427 (P₁) and F₁ generation of four different crosses

Generation	Pl. No.	Av. Pollen fertility (%)	Av. Seed set per spike	Pl. No.	Av. Pollen fertility (%)	Seed set per spike
Sel.2427	1	87.16	22.00	2	88.77	24.00
	3	88.81	28.50	4	85.36	30.17
	5	85.27	24.17	6	92.57	31.56
	7	86.13	26.67	8	91.20	26.56
	9	92.33	27.00	10	79.61	22.56
	11	91.17	29.75	12	87.19	29.40
	Mean	87.96	26.86	Range	79.61-92.57	22.00-31.56
Sel.2427 X Agra Local	1	52.68%	7.83	2	35.56%	8.00
	3	65.15%	6.5	4	36.86%	4.25
	5	38.56%	8.00	-	-	-
Agra Local X Sel.2427	1	51.93%	4.00	2	63.22%	2.00
	3	26.20%	4.80	4	65.15%	6.50
	5	54.48%	4.25	-	-	-
Sel.2427 X NI 5439	1	73.46%	9.92	2	35.28%	3.00
	3	43.50%	8.67	-	-	-
NI 5439 X Sel.2427	1	66.87%	9.37	2	59.70%	9.13
	3	55.12%	4.50	-	-	-
	Mean	51.48	6.30	Range	26.20 -73.46	2.00-9.92

Table 5: Joint segregation analysis between leaf rust resistance gene and SSR markers in F₂ population from the cross Agra Local X Sel. 2427 (I) and Sel. 2427 X Agra Local (II)

Marker	Population	Resistant Plants			Susceptible plants			Total plants	Joint Segregation	
		MM	Mm	mm	MM	Mm	mm		χ^2 Value	P Value
Xwmc527	I	39	49	29	4	14	16	151	14.033	0.0154
	II	34	49	15	3	9	13	123	17.358	0.0039
Xgwm547	I	28	75	8	7	10	17	145	32.453	<0.0001
	II	37	49	7	3	10	12	118	27.876	<0.0001
Xwmc632	I	46	68	4	10	15	9	152	34.825	<0.0001
	II	57	37	4	12	10	3	123	74.659	<0.0001
Xgwm340	I	22	72	19	6	12	16	147	17.317	0.0039
	II	33	48	17	4	8	13	123	14.930	0.0107
Xgpw4044	I	99		19	15		19	152	21.193	<0.0001
	II	85		13	10		15	123	22.359	<0.0001

190x107mm (300 x 300 DPI)

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Online Resource 1: Result of Single Race Testing (SRT) in Sel. 2427

Pathotypes designation		Disease reaction on parents (ITs)			Pathotypes designation		Disease reaction on parents (ITs)		
Old	New	S 2427	Agra Local	NI 5439	Old	New	S 2427	Agra Local	NI 5439
11	0R8	1 ⁺	3 ⁺⁺	33 ⁺	77-2	109R31-1	11 ⁺	3	3
12A	5R13	;1 ⁻	33 ⁺	33 ⁺	77-3	125R55	1 ⁻	3	3
12-1	5R37	1	3	3	77-5	121R63-1	;1	3	3 ⁻
12-4	69R13	;	33 ⁺	33 ⁺	104A	21R31	1 ⁺	33 ⁺	;1 ⁺
12-7	93R45	;11 ⁺	3	3	104B	29R23	;1	3	3 ⁼
12-8	49R45	1 ⁺	3	3	104-1	21R31-1	;1 ⁻	33 ⁺	NG
12-9	93R37	2 ⁺	3	3	104-2	21R55	;1	3	3
162	93R7	;1	3	3	104-3	21R63	;1 ⁼	3	11 ⁺
162-2	93R39	1	3	3 ⁻	104-4	21R57	1 ⁺	3 ⁺	3
77	45R31	;1	3	;1	106	0R9	1 ⁺	3	3 ³
77A	109R31	1	3 ⁺	3	107-1	45R35	;	3 ⁺	3 ³
77A-1	109R23	;1	33 ⁺	3 ⁻	108-1	57R27	0;	3	;
77-1	109R63	11 ⁺	3	3					

Note: IT means Infection Types; NG-Nil Germination

190x107mm (300 x 300 DPI)