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ORIGINAL ARTICLE

An approach for high-resolution genetic mapping of distant wild relatives of bread wheat: example of fine mapping of *Lr57* and *Yr40* genes

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Abstract

Key message **The article reports a powerful but simple approach for high-resolution mapping and eventual map-based cloning of agronomically important genes from distant relatives of wheat, using the already existing germplasm resources.**

Abstract Wild relatives of wheat are a rich reservoir of genetic diversity for its improvement. The effective utilization of distant wild relatives in isolation of agronomically important genes is hindered by the lack of recombination between the homoeologous chromosomes. In this study, we propose a simple yet powerful approach that can be applied for high-resolution mapping of a targeted gene from wheat's distant gene pool members. A wheat-*Aegilops geniculata* translocation line TA5602 with a small terminal segment from chromosome 5 M^g of *Ae. geniculata* translocated to 5D of wheat contains genes *Lr57* and *Yr40* for leaf rust and stripe rust resistance, respectively. To map these genes, TA5602 was crossed with a susceptible *Ae. geniculata* 5 M^g addition line. Chromosome pairing between the 5 M^g chromosomes of susceptible and resistant parents resulted in the development of a high-resolution mapping panel for the targeted genes. Next-generation-sequencing data from flow-sorted 5 M^g chromosome of *Ae. geniculata* allowed us to generate 5 M^g-specific markers. These markers were used to delineate *Lr57* and *Yr40* genes each to distinct ~ 1.5 Mb physical intervals flanked by gene markers on 5 M^g. The method presented here will allow researchers worldwide to utilize existing germplasm resources in genebanks and seed repositories toward routinely performing map-based cloning of important genes from tertiary gene pools of wheat.

Introduction

Bread or hexaploid wheat (*Triticum aestivum* L; $2n = 6x = 42$; *AABBDD*) is one of the most important staple crops and provides over one-fifth of the calories consumed by the world's population (Shewry and Hey 2015; Shiferaw et al. 2013). Wheat production and global food security face a number of challenges including rising global temperatures, increasing human population, deterioration of natural resources and several biotic stresses (Godfray et al. 2010; Chen et al. 2014). Genetic variation is an indispensable tool that plant breeders must access to develop varieties that can tackle biotic and abiotic stresses. *T. aestivum,* arose from a rare spontaneous hybridization event between a tetraploid species, *T. turgidum* ($2n = 4x = 28$; *AABB*), and a diploid species *Aegilops tauschii* ($2n = 2x = 14$; *DD*) (Kerber 1964; Huang et al. 2002). Thus, the total amount of genetic variation within domesticated wheat is largely limited to the founder genotypes of the original hybrids (Ladizinsky

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1985). Although cultivated wheat has a narrow genetic base, its wild and related germplasm provides a rich resource to introduce genetic diversity in wheat and other Triticeae crop plants (Mondal et al. 2016; Jiang et al. 1993).

The utility of wild species as a source of genetic variation was recognized over 100 years ago, and due to that reason out of the 410,000 wheat accessions housed among the worldwide gene bank collections, approximately 60% are wild species (Tanksley and McCouch 1997). Triticeae members that can contribute genetic material for wheat improvement have been classified as primary, secondary, or tertiary gene pools based on their genome commonality with the bread wheat genome (Harlan and de Wet 1971; Jiang et al. 1993). These gene pools serve as excellent sources of genetic variability to improve wheat varieties. Primary gene pool members include cultivated varieties and landraces of hexaploid and tetraploid wheat, as well as the diploid *Ae. tauschii* (D genome). Recombination can occur between chromosomes of these species and therefore, primary gene pool members have been widely explored for wheat improvement (Jiang et al. 1993; Friebe et al. 1996). However, tremendous untapped genetic diversity exists among the secondary and tertiary gene pool members of wheat (Jiang et al. 1993; Rasheed et al. 2018). Species from the secondary gene pool are mostly polyploids that share one of their genomes with wheat (A or D). Genes contained within homologous genomes can be introduced through recombination, but genes in nonhomologous (referred to as homoeologous) chromosomes do not normally pair without chromosome engineering approaches (Sears 1972; Friebe et al. 1996; Qi et al. 2007; Niu et al. 2011). The tertiary gene pool is comprised of species having no genome in common with wheat, and thus their chromosomes do not recombine with those of wheat (Friebe et al. 1996; Feuillet et al. 2008; Tiwari et al. 2015). Homoeologous chromosome pairing between wheat and alien chromosomes can be induced; however, its frequency is very low (~0.1–2%) (Qi et al. 2007).

Introgression of alien genetic material into wheat has a long history and an enormous amount of genetic diversity is available in the form of wheat–alien introgression lines (Gill et al. 2011). These introgression lines containing specific target traits were generated by systematic and strenuous work of many geneticists and breeders across the globe over the past century (Gill and Raupp 1987; Gill et al. 2006, 2011). Species from at least 25 genera of Triticeae tribe have been successfully crossed with wheat and are the source of the introgression lines (Jiang et al. 1993; Gill et al. 2006, 2011; King et al. 2019). The F_1 hybrids can often be manipulated to retain entire alien chromosomes in wheat and large translocations can be induced by chromosome engineering (Qi et al. 2007). For example, the Wheat Genetic Resources Center (WGRC) at Kansas State University presently maintains 231 alien substitution lines and 370 alien addition lines as well as 159 wheat lines carrying translocations from seven different species (Gill et al. 2006). This is just one example from WGRC, several other gene banks such as Kyoto University—Japan, John Innes Centre—UK, CIMMYT wide crossing program—Mexico, University of Adelaide—Australia, Punjab Agricultural University—India, and Nanjing Agriculture University—China house hundreds of such germplasm (Feuillet et al. 2008; Ortiz et al. 2008a; Awlachew et al. 2016; Rasheed et al. 2018; King et al. 2019; Narang et al. 2020). However, due to the large size of most of these introgressions, considerable linkage drag is associated with the alien chromatin in the introgression and translocation lines (Payne 1987; Burnett et al. 1995; Clarke et al. 1996). Highly suppressed recombination between wheat and alien chromosome(s)/chromosome segment(s) limits their use in precise gene mapping and cultivar development (Qi et al. 2007; Summers and Brown 2013). Precise mapping of genes is critical in identification and better utilization of the genetic diversity from the wild relatives of wheat. Development of an approach that can allow recombination within the identified alien segment will help tremendously in precise mapping and map-based cloning of agronomically important genes from the distant gene pool of wheat.

Large volumes of alien chromosome-specific markers are necessary for efficient utilization of wild wheat relatives, especially for fine-scale mapping and to identify small and useful translocations. Recent advances in the wheat reference genome and transcriptome, low-cost next-generation sequencing technologies, and high-throughput genotyping platforms provide efficient solution to this limitation. Single-nucleotide polymorphism (SNP) markers have become the technology of choice for all organisms because of their wide distribution in genomes and compatibility with high-throughput, multiplex detection platforms (Cavanagh et al. 2013; Wang et al. 2016; Cseh et al. 2019). Previously, we developed a pipeline for alien chromosome-specific SNP discovery in wheat (Tiwari et al. 2014). Utilizing the advances in chromosome sorting approaches, we flow-sorted a complete alien chromosome (5 M^g from *Ae. geniculata*), followed by its sequencing, and showed the use of marker resources in detecting small to large 5 M^g-specific introgressions having useful genes (Tiwari et al. 2014, 2015). Short read sequence assemblies for alien chromosomes provide a useful resource for developing molecular markers, mapping and their usage in breeding programs (Tiwari et al. 2014, 2015; Grewal et al. 2020).

In the present work, we propose a strategy to escape the bottleneck of limited recombination between wheat and alien chromatin for genetic mapping of useful genes from distant relatives of wheat. Using the examples of leaf rust resistance gene *Lr57* and stripe rust resistance gene *Yr40* from *Ae. geniculata*, we created a mapping population by crossing resistant wheat-*Ae. geniculata* translocation line with

a susceptible *Ae. geniculata* disomic 5 M^g addition line. Genomic resources developed for 5 M^g by us, in conjunction with the IWGSC wheat reference sequence, enabled 5 M^g-specific marker development, making genetic mapping in tertiary gene pool members possible. The presented strategy will enable mapping and identification of useful genes from distant wild relatives of wheat, from which large translocations have been considered as genetic dead-ends so far.

Material and methods

Plant material and development of mapping population

TA5601 and TA5602 are resistant *Ae. geniculata* translocation lines (T5DL.5DS-5M^gS) having leaf rust and stripe rust resistance genes *Lr57* and *Yr40*. TA7659 is a leaf rust and stripe rust susceptible disomic addition line of chromosome 5 M^g in Chinese Spring background. Table 1 provides a detailed description of all the plant materials used in this study. To allow genetic recombination in the *Lr57* and *Yr40* regions, we crossed TA5602 with TA7659. The F_1 plants were grown from the crossed seeds (between TA5602 X TA7659), and F_2 seeds from the selected plants (showing trivalents) were grown to develop an F_2 population of 162 lines used in this study for genetic mapping of the targeted traits. Leaf rust phenotypes were subsequently confirmed using 116 $F_{2:3}$ families, and stripe rust phenotypes were evaluated on a copy of 112 $F_{2:3}$ families.

Cytological pairing evaluations of F_1 hybrids

For meiotic analysis, developing spikes of F_1 plants were fixed in Cornoy's solution (6 ethanol:3 chloroform:1 acetic acid) for 24 h and transferred to 70% ethanol (Tiwari et al. 2010). Anthers at various stages of meiotic division I were squashed in 2% acetocarmine, and the pollen mother cells (PMCs) were scored for chromosomal pairing in all the crosses. Photographs were taken with a digital camera (Canon PC1049, No. 6934108049).

Developing 5 M^g-specific PCR based markers, characterization of translocation sizes, and genotyping of mapping panel

Chromosome 5 M^g from rust-resistant *Ae. geniculata* accession TA10437 was previously flow-sorted and shotgun sequenced (Tiwari et al. 2015). In the present work, wheat 5D reference was used as the starting point for 5 M^g-specific primer design, because *Ae. geniculata* chromosome 5 M shares high synteny and collinearity with 5D (Tiwari et al. 2015), but 5 M^g itself does not have complete reference sequence. Our approach was to use 5 M^g sequence with high 5DS homology to localize 5 M^g sequences to our region of interest. Wheat 5DS gene sequences spanning 0–40 Mb were selected from the High Confidence Genes v1.1 IWGSC to serve as queries for command line BLAST against 5 M^g scaffolds derived from TA10437 (Tiwari et al. 2015). The wheat 5D and 5 M^g assembly matches and high-scoring BLAST sequence matches from wheat 5A and 5B were aligned using Genome specific primer design software GSP (Wang et al. 2016) to design 5 M^g-specific markers. Reference sequence of *Ae. tauschii*, the D genome donor of wheat (Luo et al. 2017), was also used to aid in removing the D genome amplifying markers. To verify 5 M^g-specificity, primers were tested in duplicate on TA10437, the introgression lines TA5602 and TA5601, WL711, and TA7659. Primers that amplified WL711 or failed to amplify TA10437 were excluded from further testing. TA7659 contains a

Table 1 List of wheat germplasm used in this study

Plant ID	Plant species	Type of germplasm	Ploidy and genome designation	Description
TA10437	*Ae. geniculata*	Wild wheat species	$2n=4x=28$; UUMM	Resistant to leaf, stripe, and stem rust diseases
TA1800	*Ae. geniculata*	Wild wheat species	$2n=4x=28$; UUMM	Susceptible to leaf, stripe, and stem rust diseases
WL711	*T. aestivum*	Hexaploid wheat variety	$2n=6x=42$; AABBDD	A susceptible spring wheat cultivar
TA5601	*T. aestivum*	Resistant translocation line in hexaploid wheat	$2n=6x=42$; T5DL.5DS-5M^gS	A Wheat-*Ae. geniculata* translocation line with ~5% of 5 M^g short arm segment translocated on 5D
TA5602	*T. aestivum*	Resistant translocation line in hexaploid wheat	$2n=6x=42$; T5DL.5DS-5M^gS	A Wheat-*Ae. geniculata* translocation line with ~20% of 5 M^g short arm segment translocated on 5D
TA7659	*T. aestivum*	Susceptible disomic addition line in hexaploid wheat DA-5 M	$2n=6\times44$; AABBDD + 5 M^g	Contains 21 pairs of wheat chromosomes and one additional pair of chromosome 5 M^g added from susceptible *Ae. geniculata*

rust-susceptible 5 M^g chromosome, and since markers were designed from resistant 5 M^g sequence assembly, both amplification and failure to produce amplicons could be expected for TA7659. Moving from the telomeric region to the centromeric region, primers would change from amplifying both introgression lines and TA10437 to only amplifying TA10437 and TA5601 (with bigger translocation segment), indicating the end of the TA5602 5 M^g translocation.

DNA extraction, PCR, and sequencing

Parental leaf tissue was collected on ice from plants of the mapping population at the seedling stage and then stored at − 80 ℃ before extraction. DNA was extracted in water using the Kingfisher Flex DNA extraction robot with the Biosprint 96 DNA extraction kit (Qiagen, Valencia, CA, USA) and then quantified. DNA samples were diluted to 25 ng/µL.

PCR was carried out using Bioline MyTaq PCR kits (Bioline, Taunton, MA, USA) in 10 µL reactions with touchdown PCR profiles of: 95 ℃—5 min, six cycles of 95 ℃—1 min, 63 ℃—1 min with a decrease of 1 ℃ per cycle and 72 ℃—2 min, followed by 25 cycles of 95 ℃—1 min, 52–56 ℃—1 min and 72 ℃—2 min, and a final extension of 72 ℃—7 min. Half of the PCR product volume was visualized on 1% agarose gels, while the second half was used for Sanger sequencing, to confirm amplification specificity to the predicted 5 M^g region. Sanger sequencing was done on the ABI3739xl (Applied Biosystems, Foster City, CA, USA).

To distinguish PCR amplification failure from dominant marker polymorphism between the introgression lines and WL711, primers of interest were also multiplexed with internal control primers during PCR that could amplify uniformly across all lines and be distinguished by size on an agarose gel. The 5 M^g primers not amplifying TA7659 and WL711 were selected as dominant markers for characterization of translocation sizes in TA5601 and TA5602 and mapping of *Lr57* and *Yr40*. If a primer pair did amplify TA7659, it was sequenced along with TA5602, TA5601 and TA10437, and the sequences were then aligned on Clustal Omega to identify any sequence polymorphism, including SNPs or deletions, and then confirmed with the trace files. These markers were then sequenced in our mapping population for mapping *Lr57* and *Yr40*.

Phenotyping of leaf rust

To select the most appropriate leaf rust race providing contrasting phenotypes on parents of the mapping population, an initial screening of the parental set along with controls (TA5602, TA5601, WL711, TA7659 and Jagger) was conducted at the seedling stage using four leaf rust races, i.e., PRTUS54, PRTUS55, PRTUS25 and PRTUS61. All plants were grown in pots with a 1:1 vermiculite–soil mixture. Ten-day-old seedlings were inoculated by spraying the seedlings with suspension of urediniospores in Soltrol 170 isoparaffin light mineral oil (Chevron Phillips Chemical Company LLC, The Woodlands, TX). Urediniospores of leaf rust cultures stored at − 80 °C were heat shocked prior to inoculation at 40 °C for 5 min. After the evaporation of the mineral oil, the inoculated seedlings were incubated in a dew chamber at 20 ± 2 °C for 16 h. After 14 days of the inoculation, the phenotypic data on disease spread were recorded using the 0–4 Stakman scale (Roelfs et al. 1992) as follows: 0 = no uredinia or other macroscopic sign of infection, ';' = hypersensitive necrotic or chlorotic flecks without uredinia, 1 = small uredinia surrounded by necrosis, 2 = small- to medium-sized uredinia surrounded by necrosis, 3 = medium- to large-sized uredinia with or without chlorosis, 4 = large-sized uredinia without chlorosis.

Phenotyping of 162 F_2 plants along with controls was conducted using the selected leaf rust race PRTUS55 following the same procedure. To reconfirm the phenotype and to identify homozygous and heterozygous resistant, and susceptible families, progeny testing of 116 $F_{2:3}$ families was conducted by phenotyping ten seeds per F_2 family as described above. WL711 and translocation line TA5602 were used as controls in all the experiments.

Phenotyping of stripe rust

All stripe rust disease evaluation experiments were conducted in the greenhouse and growth chamber under controlled conditions. First, the parental set (TA5601, TA5602, TA7659 and WL711) were tested for their stripe rust response at seedling stage. For parental testing, two replications of five plants per accession were tested with stripe rust race AR90-1. Plant growth conditions and inoculation setup were same as that described for leaf rust phenotyping, except that the stripe rust inoculated seedlings were incubated for 20 h in a dew chamber at 12 ± 2 °C. After incubation, the seedlings were transferred to growth chamber, with temperature setting of 12 ℃ at night and 15 ℃ at day with 16 h of photoperiod. Infection types (ITs) were recorded 18–21 days post inoculation, using the 0–9 (McNeal et al. 1971). Plants having infection types 0–2 were considered as resistant, those having infection types 3–5 with intermediate resistance and those scored between 6 and 9 ranged from susceptible to highly susceptible.

A set of 112 $F_{2:3}$ of TA7659 X TA5602 families was phenotyped at seedling stage using the protocol described above. Ten seeds of each $F_{2:3}$ family as well as parental lines were grown in pots and were inoculated with stripe rust race AR90-1 at two-leaf stage. Data recording was done according to method described above.

Cytological confirmation of recombination between susceptible and resistant *Ae. geniculata* 5 M^g segments with GISH and FISH

Recombination in the $F_{2:3}$ families was also confirmed using genomic in situ hybridization (GISH) and fluorescent in situ hybridization (FISH) of plants selected on the basis of molecular markers. Preparations of mitotic chromosomes for GISH and FISH were done according to (Koo et al. 2017). The genomic DNA of *Ae. comosa* ($2n = 2x = 14$; MM) was used to paint 5 M^g chromosome segments for GISH, whereas pAS1 probe, known to exclusively label the D genome chromosome, was used for FISH (Zhang et al. 2001). Probes were labeled with either digoxigenin-11-dUTP (pAS1) or biotin-16-dUTP (*Ae. comosa*), according to the manufacturer's instructions (Roche). Unlabeled total genomic wheat DNA was used as a blocker. After post-hybridization washes, the probes were detected with Alexafluor 488 streptavidin (Invitrogen, Grand Island, NY) for biotin-labeled probes and rhodamine-conjugated anti-digoxigenin (Roche, Indianapolis, IN) for dig-labeled probes. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in Vectashield antifade solution (Vector Laboratories, Burlingame, CA). The images were captured with a Zeiss Axioplan 2 microscope using a cooled CCD camera CoolSNAP HQ2 (Photometrics, Tucson, AZ) and AxioVision 4.8 software (Carl Zeiss Microscopy LLC, Thornwood, NY). The final contrast of the images was processed using Adobe Photoshop CS5 software. Chromosome measurements were done by Image J software.

Mapping of the *Lr57* and *Yr40* genes

Markers confirmed to be polymorphic were used to genotype the TA7659xTA5602 mapping population. Since dominant 5 M^g resistant markers could not distinguish between homozygous and heterozygous individuals, sequencing based polymorphic markers were used to differentiate the zygosity of the lines. Genotyping data were then used to create a genetic map by R qtl, using hidden Markov models to account for the genotyping information of dominant markers. Genetic distances were calculated with the Kosambi mapping function (Kosambi 1943; Broman et al. 2003). Map chart was used to draw the maps (Voorrips 2002).

Results

Characterization of the 5 M^g translocation lines

Translocation lines TA5602 and TA5601 carrying leaf rust (*Lr57*) and stripe rust (*Yr40*) resistance genes were previously characterized using RFLP markers to have ~ 5% and ~ 25% of the short arm of chromosome 5 M^g by Kuraparthy et al. (2007). In the present work, first we characterized the exact breakpoints of 5 M^g segments in the resistant translocation lines to define the mapping interval for *Lr57* and *Yr40*. To detect the breakpoints or physical sizes of the translocation lines, we developed 23 5 M^g-specific markers on the wheat-*Ae. geniculata* translocation lines (TA5601, TA5602). The set also included susceptible wheat line WL711, reference wheat Chinese Spring, resistant *Ae. geniculata* parental accession, and susceptible wheat-*Ae. geniculata* whole chromosome addition line for chromosome 5 M^g (TA7659) (Table 2).

Table 2 Amplification patterns of markers from 0.97 to 60 Mb on translocation lines TA5602, TA5601 and control lines TA10437 and WL711. All primers that amplify TA5602 and TA5601 also amplify TA10437. None of the primers amplify WL711, suggesting 5 M^g-specificity. '+' and '−' indicate presence or absence of amplification

		Position of 5M ^{g}-specific markers																							
Germplasm Identity	Detail of the germplasm	5M^g_0.97Mb	5Mg_1.5Mb	5Mg_1.8Mb	5Mg_3.6Mb	5Mg_3.9Mb	5Mg_4Mb	5Mg_4.2Mb	5Mg_4.3Mb	5Mg_5.5Mb	5Mg_5.6Mb	5Mg_6.0Mb	5Mg_7.4Mb	5Mg_7.8Mb	5Mg_8.0Mb	5Mg_8.9Mb	5Mg_9.4Mb	5Mg_9.5Mb	5Mg_9.7Mb	5Mg_9.9Mb	5Mg_17.0Mb	5Mg_35.0Mb	5Mg_45.0Mb	5Mg_50.0Mb	5Mg_60.0Mb
TA10437	Resistant *Ae. geniculata* accession	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TA5601	Resistant T5DL.5DS-5MgS (0.75)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
TA5602	Resistant T5DL.5DS-5MgS (0.95)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–
WL711	Susceptible bread wheat variety	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Physical size of 5 M^g translocated fragment in TA5602

The 5 M^g-specific markers spanning 0.97 Mb to 17 Mb were used to characterize the size of 5 M^g translocation in TA5602. Multiplex PCR was used with additional primers as internal controls in all PCR reactions to rule out misjudgment of PCR failure as the absence of marker amplification. Markers from 0.97 Mb up to 9.4 Mb consistently amplified TA5602 and TA5601, but not WL711 (Fig. 1a). However, marker at 9.50 Mb did not amplify in TA5602, WL711 and Chinese Spring. All the markers also consistently amplified TA10437 and TA5601 demonstrating that there is a breakpoint between 5 M^g and 5D chromosome between 9.4–9.5 Mb in TAA5602. Based on the amplification patterns, we concluded the size of the 5 M^g segment in the translocation line TA5602 to be 9.4–9.5 Mb (Table 2). Corresponding region on chromosome 5D was found to contain 166 high confidence annotated genes in the wheat reference sequence (IWGSC 2018).

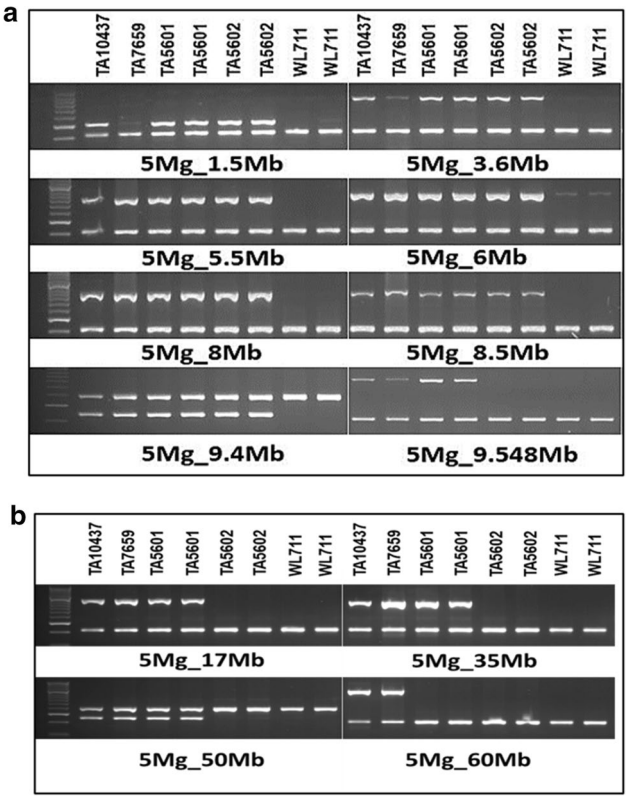

Fig. 1 Characterizing the 5 M^g introgression size in: **a** TA5602 and **b** 5601 with 5 M^g-specific primers. Markers up to 9.4 Mb amplify in TA5602, whereas markers up to 50 Mb consistently amplify in TA5601. All 5 M markers produce amplicons resistant Ae. geniculata TA10437. The band consistently present in all the lanes corresponds to internal control amplicons used to rule out PCR failure

Physical size of 5 M^g translocated fragment in TA5601

Since earlier studies identified that the 5 M^g segment in TA5601 is about 25% of the short arm, we designed the markers at every 10 Mb interval and tested on the same set of previously described lines. We found that the 5 M^g-specific markers did not amplify in TA5601 beyond 60 Mb region with respect to 5D chromosome of bread wheat (Fig. 1b), suggesting that the size of 5 M^g segment in TA5601 is between 50–60 Mb. Table 2 summarizes the amplification results for characterization of TA5602 and TA5601 translocation segment lengths and identifies exact breakpoints between 5 M^g and 5D chromosomal regions in the targeted translocation lines TA5602 and TA5601, respectively.

Leaf rust and stripe rust resistance patterns of 5 M^g translocation lines

Testing of the translocation lines with four different leaf rust races PRTUS54, PRTUS55, PRTUS25 and PRTUS61 showed *Lr57* to provide a broad-spectrum resistance against all four races (Supplementary Table SI 1), validating the findings of Kuraparthy et al. (2007). Stripe rust phenotyping with race AR90-1 showed *Yr40* to provide an immune response in both TA5602 and TA5601. Wheat-*Ae. geniculata* disomic addition line (TA7659), and hexaploid bread wheat lines WL711 and Chinese Spring were highly susceptible to both rust diseases for the tested races. Since TA5602 had a smaller 5 M^g fragment (less than 10 Mb) and it showed presence of both *Lr57* and *Yr40* genes, TA5602 was selected as resistant parent to develop the high-resolution genetic mapping population. We also screened TA7659 (with 5 M^g pair added from a susceptible *Ae. geniculata* accession) with these races of leaf rust and stripe rust and found it to be susceptible for all the tested races (Fig. 2). TA7659 was used as the susceptible parent of the mapping population.

Cytological analysis of 5 M^g chromosome pairing behavior

Cytological observations at meiotic metaphase-1 in Pollen Mother Cells (PMCs) of F_1 plants were made to study the pairing behavior of chromosomes. Normally, in bread wheat, PMCs at meiosis have 42 chromosomes arranged as 21 bivalents (in a ring/rod-like structure showing pairing between the homologous chromosomes). Since the ~ 5% chromosome 5 M^g segment translocated in TA5602 would recombine with the 5 M^g chromosome of TA7659, whereas the remaining ~ 95% of the wheat 5D chromosome in TA5602 would pair with wheat 5D homoeologue from TA7659, the chromosomes at meiotic metaphase-1 pairing are expected to be arranged in trivalents, unlike normal wheat chromosomes in a bivalent configuration (Fig. 3a). In the F1 (TA7659

X TA5602) hybrids, 43 chromosomes with different pairing configurations were observed in each PMC, which is expected because of the additional 5 M^g susceptible chromosome contributed by the disomic addition line TA7659 parent. The availability of 5 M^g susceptible and resistant chromosome segments within the same PMC allowed the expected pairing in the region of interest. Eighty-two percent of the PMCs showed presence of trivalents (Fig. 3b). Average pairing frequency data from 25 PMCs each from the six F_1 plants are provided in Table 3. Occurrence of trivalents in the F_1 plants suggested potential genetic recombination between the 5 M^g segments from the resistant and susceptible parents, indicating that the mapping strategy should be successful.

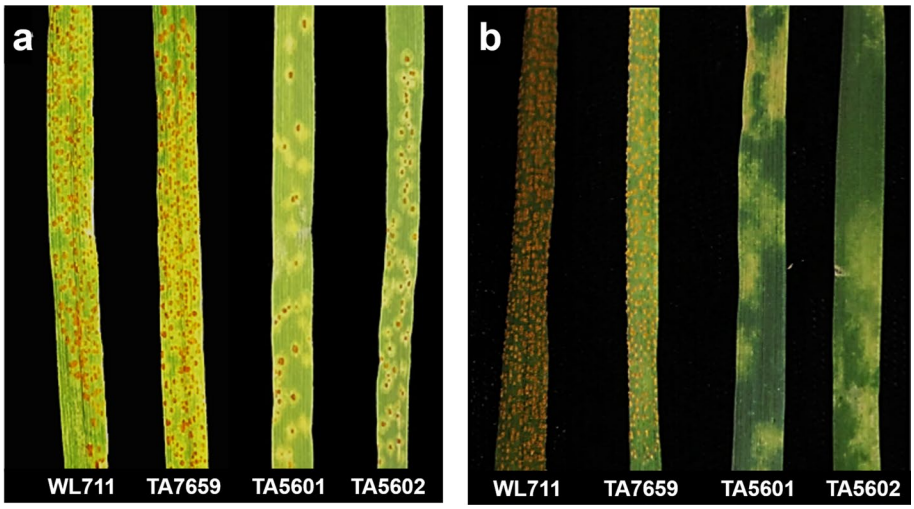

Fig. 2 Response of translocation lines TA5601, TA5602, addition line TA7659 and wheat variety WL711 to: **a** Leaf rust and **b** Stripe inoculations at seedling stage. **a** TA5602 and TA5601 show moderate to high resistance response, while WL711 and TA7659 show susceptible response with Leaf rust race PRTUS55. **b** TA5602 and TA5601 show immune response and WL711 and TA7659 show susceptible response to stripe rust race AR90-1

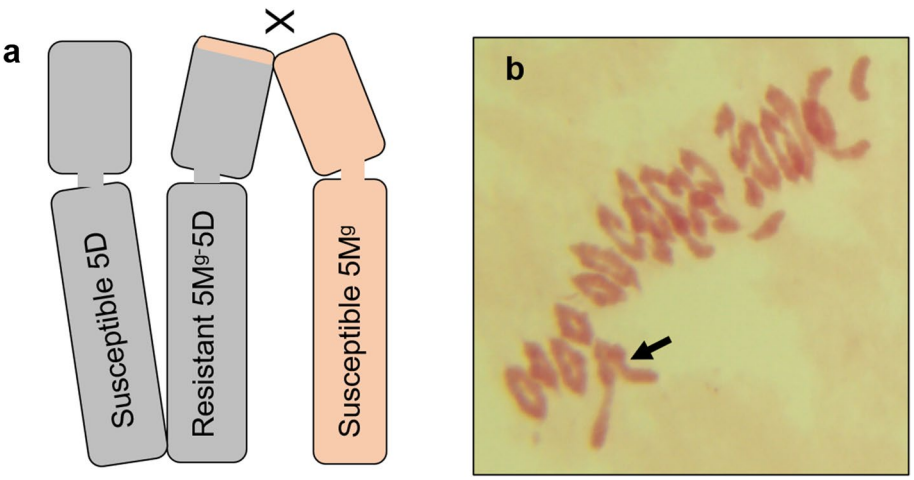

Fig. 3 Chromosome pairing in the 5 M^g segment of TA5602 and TA7659 5 M^g chromosome in the F_1 TA7659X TA5601 plants: **a** Diagrammatic representation of expected pairing behavior to show trivalent formation, 'x' indicates pairing **b** Chromosomes arranged in Metaphase plate during Meiosis-I. Black arrow indicates trivalent suggesting potential pairing of translocation chromosome from TA5602 with both 5D and 5 M^g chromosomes of TA7659. Other chromosomes are visible as ring or rod bivalents

Table 3 Chromosome number and average pairing data of selected F1 plants (TA7659 X TA5602)

F1 plant ID	2*n*	Univalents mean* ± SD**	Bivalents mean ± SD	Trivalents mean ± SD
TA7659 X TA5602-P-1	43	1.12 ± 0.33	19.06 ± 0.26	0.94 ± 0.19
TA7659 X TA5602-P-2	43	3.86 ± 0.15	18.29 ± 0.21	0.86 ± 0.44
TA7659 X TA5602-P-3	43	2.57 ± 0.29	19.11 ± 0.35	0.76 ± 0.23
TA7659 X TA5602-P-4	43	3.79 ± 0.50	18.35 ± 0.58	0.83 ± 0.35
TA7659 X TA5602-P-5	43	1.36 ± 0.31	19.52 ± 0.15	0.89 ± 0.29
TA7659 X TA5602-P-6	43	1.53 ± 0.18	19.75 ± 0.34	0.66 ± 0.36

*Average of 25 pollen mother cells in each F1 hybrid plant, **Standard deviation among the 25 pollen mother cells in each F1 hybrid plant

The F_1 plants confirmed for the presence of trivalents were selfed to produce F_2 mapping population. Our expectation was that the F_2 population resulting from the F_1 TA7659 x TA5602 plants would segregate for the *Lr57* and *Yr40* region from TA5602 and TA7659 and will allow the mapping of 5 M^g-specific markers to deduce a map order. A population of 162 F_2 individuals were grown in greenhouse along with controls and parental checks and were evaluated to measure recombination frequencies and mapping potential of this approach.

Cytological confirmation of recombination between susceptible and resistant *Ae. geniculata* 5 M^g segments with GISH and FISH

To cytologically confirm recombination between the resistant and susceptible 5 M^g chromosome segments, GISH and FISH analysis was conducted on three $F_{2:3}$ individuals showing recombination with molecular markers. Figure 4 shows the GISH and FISH patterns of the recombinants along with the parents. Susceptible addition line TA7659 showed the presence of both whole 5D (Fig. 4a) and 5 M^g (Fig. 4b) chromosomes. Resistant translocation line TA5602 showed only 5% of chromosome 5 M^g translocated terminally on 5D chromosome (Fig. 4c). The recombinants, on the other hand, showed different sizes (25%, 15%, 10%) of 5 M^g translocation segments verifying the occurrence of physical recombination between 5 M^g resistant and susceptible chromosomes (Fig. 4d–f). All individuals contained 42 chromosomes with homozygous translocation segments of varying lengths (Fig. 4g–j).

Genotyping and phenotyping of the 5 M^g mapping population

To genotype the F_2 mapping population, markers that could differentiate 5 M^g of *Ae. geniculata* from group 5 chromosomes of wheat (5A, 5B, 5D), while also showing polymorphism between 5 M^g from the resistant parent TA5602 and the susceptible parent TA7659 were selected. A total of 16 polymorphic markers were used to map the 9.4 Mb physical region of *Ae. geniculata* 5 M^g in TA5602 having the genes *Lr57* and *Yr40*.

The F_2 population was screened for seedling-based leaf rust assays with the leaf rust race PRTUS55. Out of 162 F_2 individuals, 122 were resistant and 40 were susceptible,

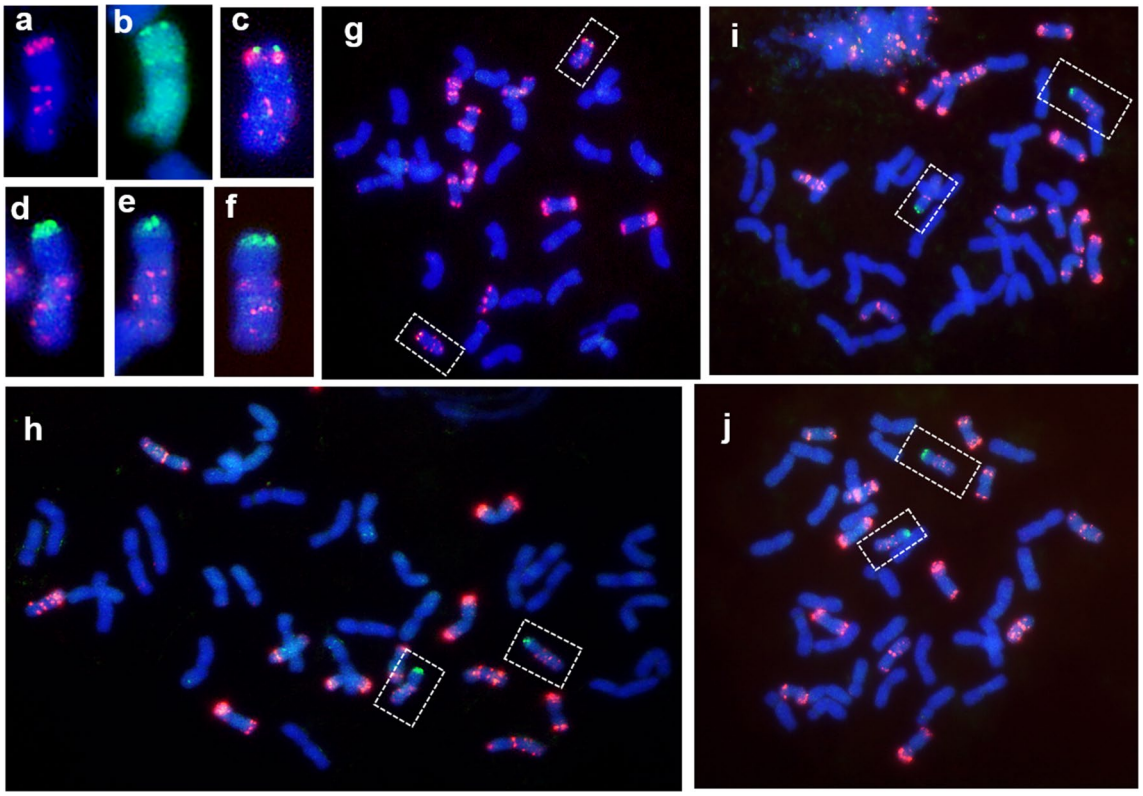

Fig. 4 GISH and FISH patterns of parents and three F3 individuals: **a** 5D chromosome of TA7659 labeled with pAS1, **b** 5 M chromosome of TA7659 labeled with *Ae. comosa* DNA, **c** Terminal 5% 5 M translocation in TA5602, **d** Recombinant-1 showing 25% of 5 M segment, **e** Recombinant-2 with 15% 5 M segment, **f** Recombinant-3 with 10% 5 M segment. Forty-two chromosomes of TA5602 **g**, Recombinant-1 h, Recombinant-2 **i**, Recombinant-3 **j**. Note the homozygous state of the translocation chromosomes in all the recombinants

fitting the 3:1 genetic ratio of single dominant gene inheritance [$\chi^2(1, n=162)=0.002$; $p=0.964329$]. After harvesting the seeds from F_2 plants, F_2-derived-F_3 ($F_{2:3}$) families having more than 20 seeds were divided in two copies, one each tested separately for leaf rust and stripe rust phenotypes. One hundred and sixteen $F_{2:3}$ families were tested for leaf rust response, out of which 32 were found to be homozygous resistant, 55 were found to be heterozygous, and 29 were found to be homozygous susceptible, fitting an expected 1:2:1 segregation ratio (χ^2 (1, $n=116$) = 0.155, $p=0.693643$). This reconfirmed that *Lr57* was inherited as a single dominant gene in the $F_{2:3}$ families. Out of the 112 $F_{2:3}$ families screened for stripe rust, 22 families were found to be homozygous resistant, 58 were found to be heterozygous, and 32 were found to be homozygous susceptible [χ^2 (1, $n=112$) = 0.0714; $p=0.0789309$]. This indicated that *Yr40* is also a single dominant gene. The ratios of inheritance for both *Lr57* and *Yr40* followed typical Mendelian segregation, confirming that recombination involving the shared 5 M^g chromosome segment in resistance and susceptible lines is occurring normally, as would happen in any other telomeric region of wheat chromosomes in the absence of the alien segments.

Lr57 and Yr40 map as two distinct loci in TA5602

Sixteen 5 M^g TA5602-specific molecular markers and two phenotypic markers generated a 23.3 cM long 5 M^g genetic map of TA5602 region, spanning the distal 9.4 Mb physical region corresponding to chromosome 5D of Chinese Spring reference wheat genome. The marker order was found to be highly conserved between 5D and 5 M^g chromosomes (Fig. 5). Our results clearly placed *Lr57* and *Yr40* as two distinct genetic loci. The leaf rust resistance gene *Lr57* from TA5602 was localized in the genetic mapping interval of 7.4–10.3 cM and was flanked by gene markers TraesCS5D01G005200 and TraesCS5D01G006600 (Fig. 5). These two markers can be used to detect the *Lr57* introgression in the crosses. Physically, *Lr57* was confirmed to be located in 3.9–5.6 Mb interval of the translocation line TA5602. Wheat chromosome 5D reference sequence contains annotated 15 high confidence genes within this interval, and they comprise the candidate genes for *Lr57* region (Table 4).

Similarly, the stripe rust resistance gene *Yr40* was localized in a 1.59 Mb physical interval toward the break point of the 5 M^g-5D translocation segment of TA5602. *Yr40* was mapped in the genetic interval of 19.9–23.3 cM and was flanked by gene markers TraesCS5D02G014100 and TraesCS5D02G016600 (Fig. 5). The wheat chromosome 5D reference sequence contains 27 high confidence genes annotated within this interval, which are the candidate genes for *Yr40* (Table 5).

Since *Aegilops tauschii* is the D genome progenitor of wheat and is a wild species, the corresponding region from 3.7 Mb to 9.7 Mb of *Ae. tauschii* 5D reference assembly (Luo et al. 2017) was also investigated for any possible additional

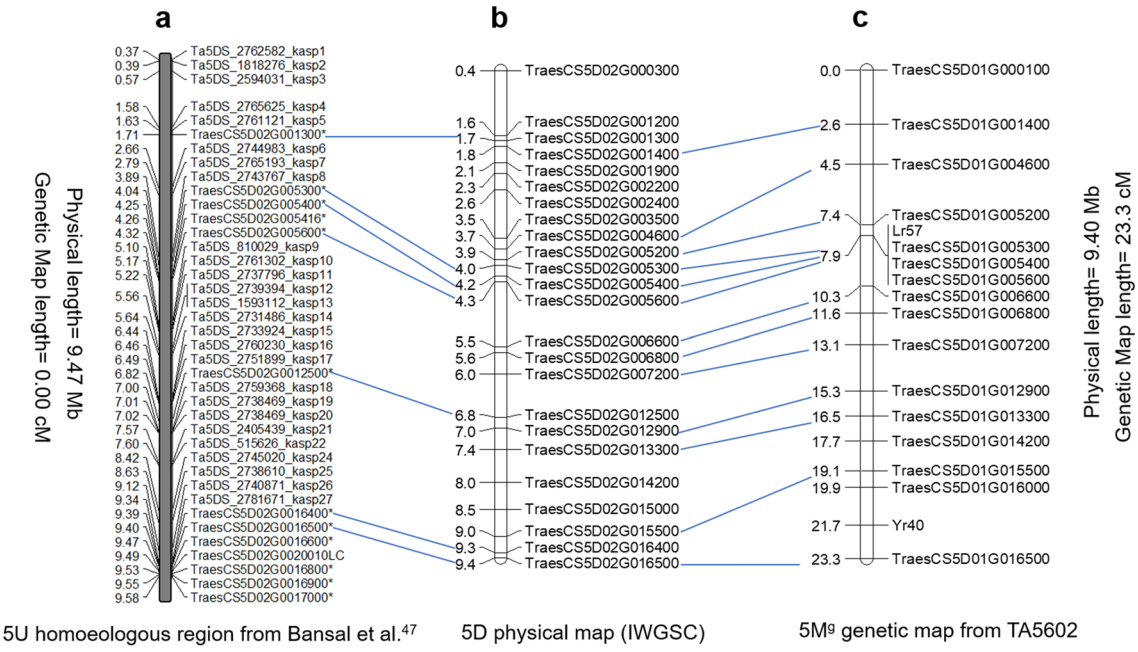

Fig. 5 Comparative mapping analysis of 5U and 5 M^g genetic map with 5D reference physical map: **a** 5U homoeologous region from Bansal et al. (2020) represented by a gray nonrecombining block, **b** Chinese Spring 5D physical map, and **c** 5 M^g genetic map showing high resolution of the corresponding region

Table 4 Fifteen putative candidate genes for *Lr57* based on the corresponding IWGSC wheat 5D chromosome assembly

IWGSC Gene ID	Description
TraesCS5D02G005200	Periplasmic serine endoprotease
TraesCS5D02G005300	Protein Kinase—nucleotide-binding leucine-rich repeat, similar to *Tsn1*
TraesCS5D02G005400	Putative disease resistance protein RGA1
TraesCS5D02G005500	Protein kinase-major sperm protein domain-containing protein
TraesCS5D02G005600	Putative disease resistance protein RGA3
TraesCS5D02G005700	Periplasmic serine endoprotease DegP-like
TraesCS5D02G005800	Probable 3-beta-hydroxysteroid-delta(8), delta(7)-isomerase
TraesCS5D02G005900	Uncharacterized mitochondrial protein AtMg00810-like protein
TraesCS5D02G006000	Ubiquitin-conjugating enzyme 15
TraesCS5D02G006100	GTP-binding brassinazole-insensitive pale green 2, chloroplastic protein
TraesCS5D02G006200	Obtusifoliol 14-alpha demethylase-like, cytochrome P450 family 51 protein
TraesCS5D02G006300	Beta-hydroxysteroid–dehydrogenase/decarboxylase-like
TraesCS5D02G006400	Obtusifoliol 14-alpha demethylase-like, cytochrome P450 family 51 protein
TraesCS5D02G006500	Obtusifoliol 14-alpha demethylase-like, cytochrome P450 family 51 protein
TraesCS5D02G006600	Cycloartenol synthase-like

Table 5 Twenty-seven putative candidate genes for *Yr40* based on the corresponding IWGSC wheat 5D chromosome assembly

IWGSC Gene ID	Description
TraesCS5D02G014100	Cytochrome P450
TraesCS5D02G014200	Cytochrome P450
TraesCS5D02G014300	Cytochrome P450
TraesCS5D02G014400	Putative ankyrin repeat protein
TraesCS5D02G014500	Putative ankyrin repeat protein
TraesCS5D02G014600	Putative ankyrin repeat protein
TraesCS5D02G014700	Putative ankyrin repeat protein
TraesCS5D02G014800	E3 ubiquitin-protein ligase RNF5-like
TraesCS5D02G014900	Hypothetical protein TRIUR3_02062
TraesCS5D02G015000	Probable phosphoinositide phosphatase SAC9
TraesCS5D02G015100	Hypothetical protein TRIUR3_02062
TraesCS5D02G015200	Elongation of fatty acids protein 3-like
TraesCS5D02G015300	Pentatricopeptide repeat-containing protein At2g38420, mitochondrial-like
TraesCS5D02G015400	CASP-like protein 4D1
TraesCS5D02G015500	Putative serine protease do-like protein
TraesCS5D02G015600	Glycosyltransferase BC10-like
TraesCS5D02G015700	Predicted protein
TraesCS5D02G015800	PROTEIN TANC2
TraesCS5D02G015900	WD repeat-containing protein 43
TraesCS5D02G016000	Peroxisome biogenesis protein 3–1
TraesCS5D02G016100	Uncharacterized protein
TraesCS5D02G016069	Wall-associated receptor kinase 2-like
TraesCS5D02G016200	Basic leucine zipper 43-like
TraesCS5D02G016300	Unnamed protein product
TraesCS5D02G016400	Von Willebrand factor type A domain-containing protein
TraesCS5D02G016500	Predicted protein
TraesCS5D02G016600	Von Willebrand factor type A domain-containing protein

genes, that may be missing in Chinese Spring assembly. Supplementary Table SI 2 provides a list of all high confidence annotated genes in the region. Twenty-six additional unique genes were found in *Ae. tauschii* 5D in the 3.7 Mb to 9.7 Mb region; however, none of them was related to disease resistance based on gene ontology/functional annotation. Interestingly, a major inversion was observed in the *Ae. tauschii* 5D assembly from 4.2 Mb to 7.4 Mb as compared to the Chinese Spring 5D reference assembly. Since the genetic map order of 5 M^g agreed with Chinese Spring reference assembly, the inversion in *Ae. tauschii* 5D sequence might be due to some assembly issues with it. More importantly, the gene order was conserved within the inversion and no additional gene related to disease resistance was observed in the *Ae. tauschii* 5D sequence, indicating that the candidate genes selected for both *Lr57* and *Yr40* should be conserved in Chinese Spring, *Ae. geniculata* and *Ae. tauschii*.

Mapping resolution

Availability of the 5 M^g genetic map spanning a 9.4 Mb physical region provided us an opportunity to calculate the resolution of the novel mapping scheme that we proposed. Using 16 markers, a genetic map length of 23.3 cM was generated for this physical interval. Figure 5 shows the mapping resolution of the 5 M^g-genetic panel. Overall map resolution, based on the genetic map length and megabases spanned on the physical map, can be calculated as 9.4 Mb/23.3 cM. Using this calculation, the average map resolution was found as 403 Kb/cM. It suggested that the map should be able to provide breakpoints between two markers physically placed at ~ 400 Kb interval. Figure 6 presents the locations of recombinants obtained in the 9.4 Mb region. At least four such physical intervals were validated (map interval 3.7–3.9 Mb, 5.6–6.0 Mb, 7.0–7.4 Mb, 9.0–9.4 Mb), and these confirmed that the average map resolution of this panel is at least ~ 400 Kb/cM. Another physical approach to look at the map resolution of a genetic population is to analyze how fine can two physically closely spaced markers be resolved in the population. Interestingly, this panel was also able to resolve several pairs of markers placed at or around 200 Kb intervals (Fig. 6), suggesting that adding more markers may help us in realizing even higher mapping resolution potential for this panel. This is a significant achievement considering the limitations in recombination-based mapping of alien germplasm.

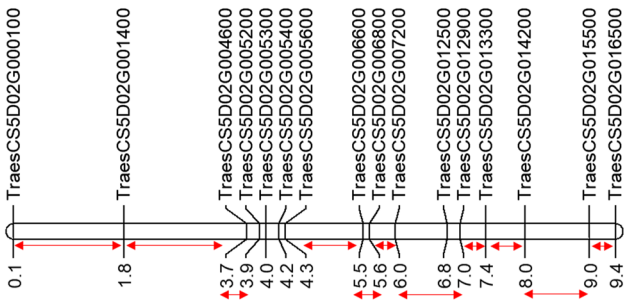

Fig. 6 Map resolution of the tested genetic mapping population. Gene identities (on the top of chromosome segment) show location of 5 M^g-specific markers on the reference 5D physical map. Map distances (below the chromosome segment) show their actual locations on the physical map in Mb. Red arrows show the regions with genetic breakpoints observed by applying 5 M^g-specific markers

Comparative mapping resolution: 5U versus 5 M

Traditionally, a selected translocation/introgression line with gene(s) of interest is crossed with an elite cultivar susceptible for the trait of interest (with or without *Ph1* system), and a large mapping population is developed with the expectation that there will be some recombination events that will allow the mapping of the targeted region. However, it is well established that the frequency of such recombination is very low (Qi et al. 2007; Wulff and Moscou 2014). In our proposed approach, a genetic mapping population can be created between a targeted translocation line carrying a gene of interest with other available translocation line for the same chromosome without the trait of interest that will allow frequent recombination events. Figure 5 shows the comparison of very similar homoeologous 5U and 5 M^g regions from two different *Aegilops* species: *Ae. umbellulata*, and *Ae. geniculata*, respectively. In an attempt to map leaf and stripe rust resistance genes *Lr76* and *Yr70* from 5U of *Ae. umbellulata*, Bansal et al. (2020) developed a large RIL population of 1,404 lines by crossing 5U-5D translocation line pau16057 with a susceptible wheat variety WL711. However, no recombination was observed in the 9.47 Mb interval and the whole 5U segment was inherited as a genetic block in the population (Bansal et al. 2020) (Fig. 5). Our approach provided more than 20 breakpoints in a very similar region of 5 M^g and was able to locate two agronomically important genes with an average mapping resolution of 400 Kb/cM (Fig. 5). We found that this mapping panel was able to resolve markers physically at ~ 200 Kb at least at three physical locations, indicating that the actual map resolution of this panel is up to 200 Kb depending upon the number of molecular markers used (Fig. 6).

This paper establishes a pipeline to fine-map candidate genes from wild relatives of wheat as an initial step toward their broader utilization. Figure 7 presents a schematic to perform high-resolution mapping of the alien segments using the already available wheat–alien introgression germplasm. It is presented in the context of the 5 M^g translocation but can be effectively applied to any wheat–alien translocation/ introgression stocks having gene(s) of interest.

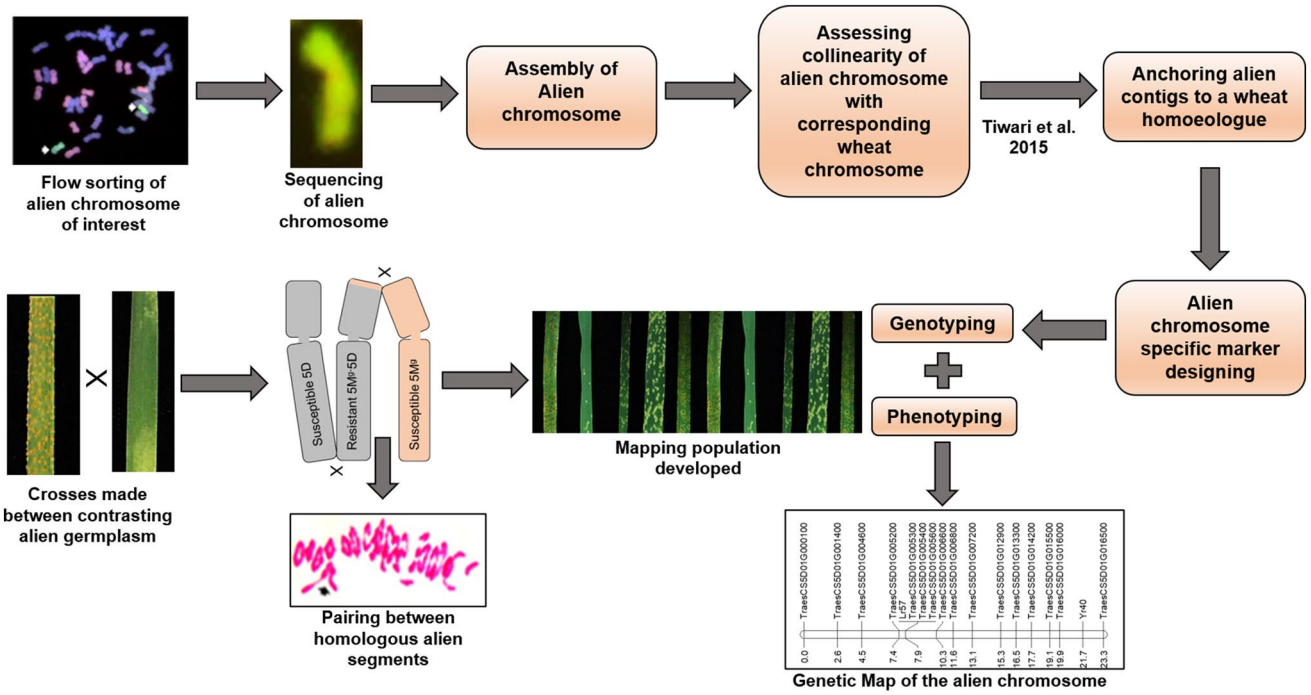

Fig. 7 A schematic to map genes in alien chromosome-specific translocation/introgression lines showing contrasting phenotypes for the targeted traits

Discussion

Utilization of germplasm resources from tertiary gene pool of wheat

Continuous introduction of novel genes and alleles in crop breeding program is a prerequisite to develop elite cultivars. This need is further exacerbated by the increasingly volatile climate, increasing human population, and dwindling natural resources (Ortiz et al. 2008b; Chen et al. 2014; Mondal et al. 2016). With very limited support for the introduction of 'genetically modified' wheat, bread wheat has to draw genetic variation from its progenitors, wild and distant relative species to enrich its genetic base and to cope with various biotic and abiotic stresses. Development of the alien introgression germplasm is a time-consuming process and require years of hard work. A number of germplasm enhancement programs worldwide led by cytogeneticists, breeders, and geneticists over the past century have produced useful germplasms by crossing wheat with its relatives from primary, secondary, and tertiary gene pools (Chen et al. 1994; Gill et al. 2006; King et al. 2019; Grewal et al. 2020). Disease resistance was the most targeted trait, however, mining was also done for a number of traits including yield components, flowering and maturity time, heat, cold and drought tolerance; grain micronutrient content; protein content and other quality traits (Jiang et al. 1993; Rawat et al. 2009; Gill et al. 2011; Wulff and Moscou 2014; King et al. 2019). Thousands of alien chromosome addition, substitution, and translocation lines for a number of species from wild relatives resulting from such efforts have been maintained in different gene banks and germplasm repositories across the globe (Tanksley and McCouch 1997; Gill et al. 2006). These precious germplasms are available on request by contacting these sources for use in wheat improvement.

In our study, we leveraged *Ae. geniculata* translocation lines TA5602 and TA5601 carrying 5 M^g fragments from *Ae. geniculata* accession TA10437. Kuraparthy et al. (2007) tested TA5601 and TA5602 (5 M^g translocations from resistant *Ae. geniculata* accession TA10437), and TA7659 (a disomic addition line with chromosome 5 M^g added from accession TA1800) for a number of leaf rust and stripe rust races (Kuraparthy et al. 2007). All the data clearly indicated that TA5602, and TA5601 showed resistant phenotype for different leaf rust races (PRTUS6, PRTUS25, PRTUS35, MCDL, PRTUS55, PRTUS54, PRTUS61) and stripe rust races (KS2005, Pst100, A90-1) whereas TA7659 was highly susceptible for all these races. Using these contrasting derivatives from the same species, we established a strategy for routinely mapping genes from distant relatives for wheat improvement. This strategy can be used in mapping genes of interest from any wild relative of wheat. For *Ae. geniculata* a number of introgression lines for each chromosomes are available from different *Ae. geniculata* accessions. This

will achieve spontaneous chromosomal pairing between the two alien segments even in the presence of *Ph1* locus and will provide a tool to map alien chromosome-specific genes and their eventual map-based cloning. A number of introgression lines have been generated for the alien chromosomes using different accessions of the same species. It might be possible that there are just one set of introgression lines available for an alien species. In such cases species-specific addition/substitution or translocation lines having similar genomes can be used to identify contrasting phenotypes and to induce pairing between alien chromosomes to precisely map an agronomically important gene. For example, there are at least three different sources of 5 M specific introgression lines available where 5 M chromosome was introgressed using different sources of different M-genome species (Molnár et al. 2005; Friebe et al. 2011; Annamaria and Molnar-Lang 2012; Liu et al. 2019; Song et al. 2020). If contrasting phenotypes are not available in chromosome 5 M of *Ae. geniculata*, then other 5 M-sources such as introgression lines from *Ae. comosa* ($2n = 2x = 14$; MM), and *Ae. biuncialis* ($2n = 4x = 28$, UUMM) could be tested to develop mapping populations.

Availability of genomic resources helps characterization of alien translocations

Availability of wheat reference genome, affordable next-generation sequencing approaches, exome-capture and marker dense-genotyping platforms has provided much-needed impetus for wheat genetics and breeding-related research. These advances have played an important role in generating genomic resources for wild and distant gene pool members of wheat. Using the flow-sorted chromosome 5 M^g sequences generated by our group (Tiwari et al. 2014, 2015) in conjunction with the wheat reference sequence (IWGSC 2018), we were able to design and validate markers specific to chromosome 5 M^g of *Ae. geniculata*. This highlights the value of comparative genomics in facilitating alien chromosome-specific primer design. Well-characterized wheat reference sequence can be aligned against less-well-characterized wild wheat relative sequences to facilitate easy primer design for alien species chromosomes. Since our earlier results indicated that chromosome 5 M^g from *Ae. geniculata* and 5D of reference wheat are highly colinear, we used 5D gene order to anchor and order 5 M^g-specific contigs and used a genome specific marker development pipeline to develop 5 M^g-specific markers (Tiwari et al. 2014, 2015). Our results based on 5 M^g/5D collinearity indicated that in the translocation line TA5602 5 M^g segment had replaced 9.4 Mb of chromosome 5D. We performed comparative genomic study based on gene-based markers and flow-sorted 5 M^g contig sequences from 0–9.4 Mb region, and it clearly indicated no gene-based deletions between 5 M^g and 5D chromosomes. Using the 5 M^g-specific markers ordered along the length of chromosome 5DS reference wheat sequence, the TA5602 introgression size was found to be between 9.4–9.5 Mb on 5DS. We now know that wheat chromosome 5D is 566 Mb, making the TA5602 introgression constitute approximately 4.75% of 5D. Similarly, TA5601 was determined to contain ~ 60 Mb of the short arm of chromosome 5 M^g of *Ae. geniculata,* which was very close to in situ hybridization-based estimations of Kuraparthy et al. (2007).

Availability of genomic resources established for the chromosome 5 M^g allowed us to develop 5 M^g-specific markers to map the targeted genes. However, with sequencing costs coming down to less than $10 per Gb and availability of tremendous resources in terms of genotyping platforms and exome-capture systems for wheat and its related species, similar resources can be quickly generated for any targeted alien chromosome(s) with agronomically important genes. We have already established and validated the pipeline for developing genomic resources for alien chromosomes using single gene FISH and comparative genomic mapping approaches (Tiwari et al. 2014, 2015; Koo et al. 2016). All the 5 M^g marker sequences used in the study are provided in Supplementary Table SI 3, and these can be used to detect 5 M^g-specific introgressions using simple PCRs and gel-based screening approaches.

Genetic mapping in alien translocations

Development of a genetic population is the first basic step in order to locate a gene controlling agronomically important phenotype(s). In majority of the wheat–alien chromosome-specific introgressions from the tertiary gene pool, pairing is not possible in the presence of *Ph1* locus, hence simple approaches do not work for generating mapping populations (Sears 1972; Qi et al. 2007; Niu et al. 2011). Chromosome engineering approaches can help to induce pairing, but these are very low resolution and time-consuming processes (Sears 1972; Qi et al. 2007). However, to map an important gene from distant gene pool, we can obtain chromosomal pairing events within an alien segment by crossing it with an introgression/translocation line of the same chromosome but from different accession with contrasting phenotype.

Since TA5602 and TA7659 showed contrasting phenotype for both the rust diseases, we crossed them to develop a mapping population. Our genotypic, phenotypic, and cytological results clearly showed that genetic recombination is taking place between 5 M^g chromosomes of resistant and susceptible parents. Using this approach, the wild-type *Ph1* locus will ensure both fertility of the progeny and the pairing and recombination occurring only between segments of homologous alien chromatin (5 M^g from TA7659) and translocation segments of chromosome 5 M^g from TA5602 on homologous wheat chromatin, allowing for the generation

of an otherwise typical F_2 mapping population (Fig. 7). Previous efforts were not able to separate *Lr57* and *Yr40* genes as separate loci and speculations about the possibility of a single gene with pleiotropic effects regulating both the traits were made (Kuraparthy et al. 2007). In the present work, using just a population of 162 F_2 plants, *Lr57* and *Yr40* loci were separated by a genetic distance of 12.6 cM in our population. Physically *Lr57* was placed between 3.9–5.6 Mb interval, whereas *Yr40* was placed in the 7.1–9.5 Mb interval. These 5 M^g translocation lines are just one example showing presence of agronomically important genes in small to large wheat–alien translocations. Such resources can be used to isolate candidate genes using positional cloning approach to enrich wheat's narrow genetic base.

Improving mapping resolution toward positional cloning of agronomically important genes from tertiary gene pool members

Several studies have shown that after integrating as a translocation segment, the alien chromatin usually does not undergo any further recombination with wheat chromosomes. For example, in a large RIL population of 1404 individuals between wheat-*Ae. umbellulata* translocation line pau16057 (carrying *Lr76* and *Yr70* genes) and susceptible wheat variety WL711, no recombination event was observed by Bansal et al. (2020). The 9.47 Mb 5U translocation in pau16057 is very similar in the size to the 5 M^g translocation in TA5602. Using our pipeline, we identified 23 recombination events in a similar 9.4 Mb region of 5 M^g translocation in TA5602, with an overall physical mapping resolution of ~ 400 Kb/cM. Using another approach to calculate the mapping resolution, based on evaluation of the ability of the mapping population to resolve two physically closely spaced markers, the mapping resolution was found to be even higher (~ 200 Kb) (Fig. 6). Although the mapping resolution obtained in this work is high, it is not unexpected, considering that the translocation segment is located at the telomeres of chromosomes 5 M. It is well documented that the telomeric regions in Triticeae are recombination hotspots (Gill et al. 1996; Akhunov et al. 2003; Saintenac et al. 2009; Koo et al. 2017). Gill et al. (1996) studied the rate of recombination in group 5 wheat chromosomes and found it to be 118 Kb/cM in the telomeric region of the chromosome, as compared to 22 Mb/cM in the centromeric regions. Since the 5 M^g translocation in TA5602 is in the telomeric end of the short arm of chromosome 5D, the high mapping resolution revalidates the concept of higher rates of recombination on the telomeric ends of the chromosome. In fact, some of the recombinants were confirmed with GISH and FISH analysis, which is a low-resolution approach; nonetheless, it clearly demonstrated recombination between the contrasting alien chromosome segments at the telomeric ends (Fig. 4).

The presented approach relies on chromosome pairing between the contrasting alien species chromosomes to facilitate recombination-based high-resolution mapping. Using this example, we show high-resolution mapping of *Lr57* and *Yr40* genes. However, it should be noted that this approach is feasible only for mapping the genes in wild species-specific genetic stocks, and not in the transfer of the chromosome segments, which is still limited by highly suppressed recombination between wheat and homoeologous alien chromosomes (Sears 1972; Qi et al. 2007; Niu et al. 2011). Spontaneous small translocations to wheat do occur, but at a very low rate (Qi et al. 2007). For the purpose of transferring an alien chromosomal segment to wheat, traditional chromosome engineering techniques using *ph1b* mutants (Qi et al. 2007; Wan et al. 2020, Dai et al. 2020) or *PhI* system (Aghaee-Sarbarzeh et al. 2000; 2002), or gametocidal gene-induced transfers (Masoudi-Nejad et al. 2002), or radiation-induced chromosome breakage techniques (Sears 1972) must be used (Wulff and Moscou 2014). High-resolution mapping of the genes of interest will provide closely spaced markers that will be critical in selecting small translocations developed using these different strategies.

Overall, this study presents a simple but powerful approach to map agronomically important genes from the tertiary gene pool of wheat, which is one of the most important resources for cereal crop improvement. This study will promote utilization of the already developed and characterized alien chromosome-specific resources in wheat by integrating this method and alien genomic resources to fine map and eventually clone valuable genes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00122-021-03851-w.

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Author contribution statement JS and TS conducted the experiments, designed markers, performed genetic analysis. BK and RB conducted leaf rust and stripe rust phenotyping of the mapping populations. DHK performed GISH-FISH analysis of recombinants. ISY performed comparative analysis with *Aegilops tauschii* assembly. BSG and PC provided germplasm material and inputs in discussion. JE provided resources for experiments. VT and NR developed the idea, planned the experiments, analyzed the results and wrote the manuscript. All co-authors read and approved the manuscript.

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Declarations

Conflicts of interest Authors have no conflicts of interest.

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