Genetic analysis of resistance to powdery mildew on 7Mg chromosome of Wheat–Aegilops geniculata,development and utilization of specific molecular markers

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Research Article

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Abstract

Background: *Blumeria graminis f. sp. Tritici* (*Bgt*) is prevalent in the main wheat-producing regions of China and result in serious yield losses in recent years. Breeding resistant cultivars is the most environmentally sound measure of disease control. *Aegilops geniculata* Roth, a close relative of common wheat, is an important and valuable disease resistance gene donor for wheat improvement.

Results: In this study, to validate powdery mildew resistance on chromosome $7M^g$, two genetic populations were constructed and analyzed. Wheat – *Ae. geniculata* $7M^g$ disomic addition line and $7M^g$ (7A) alien disomic substitution line crossed with susceptible Yuanfeng175 of susceptible powdery mildew respectively to form generations F_1 and F_2. Cytological examination, *in situ hybridization* (ISH), and functional molecular markers analysis showed that alien chromosomes could be inherited stably, produce different gamete types and enrich the intermediate materials for wheat genetic variation. The populations were inoculated with the physiological race E09 of powdery mildew at seedling stage. The results revealed that the plants showed high resistance to powdery mildew with chromosome $7M^g$. Besides, more specific markers were developed to verify chromosome $7M^g$ resistance based on SLAF-seq technique. Then, 84 specific molecular markers were obtained about chromosome $7M^g$. Among them, four markers were selected randomly to checked in two genetic populations. In summary, the above analysis confirmed that a dominant high powdery mildew resistance gene inherited were located on the chromosome $7M^g$ of *Aegilops geniculate*.

Conclusions: The results provide a basis for resistance gene mapping and specific marker development in future.

Background

Wheat(*Triticum aestivum* L., 2n = 6x = 42, AABBDD)is the cultivated cereal crops with the widest distribution, the largest cultivated area, and the highest total output in the world, and the most abundant food processing types, with 35% ~ 40% of the world population taking it as the main rations[1, 2]. However, with the change of climate and environment and the occurrence of diseases, the productivity of wheat is seriously threatened [3–5]. *Blumeria graminis f. sp. Tritici* (*Bgt*) is one of the most harmful foliar diseases of wheat worldwide, which is mainly distributed in cool and marine climate areas. Powdery mildew caused by it would seriously lead to the loss of wheat yield once the epidemic occurs [6, 7]. At present, a series of powdery mildew resistance genes, such as *Pm51*, *Pm55*, *Pm57*, *Pm58* and *Pm66*[8–12], have been identified from related species of wheat for wheat resistance improvement. Nonetheless, as the environment changes and powdery mildew will continue to mutate, these antigens will eventually gradually lose their original resistance. Therefore, it will be necessary to mine and identify more new powdery mildew resistance genes in wheat production [9–11, 13–17].

Aegilops geniculata Roth (2n = 2x = 28, $U^gU^gM^gM^g$) is an annual allotetraploid of *Aegilops* sect. *Aegilops*. Its chromosome configuration is derived from the diploids *Ae. comosa* Sm (2n = 2X = 14, MM) and *Ae. umbellulate* Zhuk. (2n = 2x = 14, UU), respectively, which has excellent traits such as resistance to diseases and insect pests, salt and alkali, and strong adaptability in long-term crop natural selection, and is mainly

distributed around the Mediterranean Sea, as well as in western and central Asia[18, 19]. Thus, *Ae. geniculata* is an important source of wild resistance genes in wheat genetics and improvement [20, 21]. The relative plant of wheat, living in the natural environment for a long time, has been tested by all kinds of terrible environmental conditions, and has a lot of excellent genes and traits which were not possessed or have been lost in common wheat. Introduction of elite genes from wheat relatives into common wheat that is an important way to increase wheat genetic diversity, broaden the genetic basis of wheat breeding and improve wheat varieties [22–25]. In 2002, Zeller et al. found the powdery mildew resistance gene *Pm29* in *Ae. geniculata* and confirmed that it was located on chromosome 7D [26]. In 2007, Kuraparthy et al. found a stripe rust resistance gene *Yr40* and a leaf rust resistance gene *Lr57* on chromosome $5M^g$ of *Ae. geniculata* [27].

In the research of wheat production, the existing molecular markers could no longer meet the rule of quickly and accurately identifying the resistance genes of related species inserted into wheat. However, the emergence of next-generation sequencing provides strong technical support for discovering wheat wild gene breeding [28, 29]. Specific-locus amplified fragment sequencing (SLAF-seq) is a simplified genome sequencing technique suitable for large-scale genotyping in the next generation sequencing, which was currently used in the development of wheat markers. For example, Wang et al. has the success rate for the development of markers specific to the Wheat– *Thinopyrum ponticum* of chromosome with a maximum success rate of 52.98% using SLAF-seq [30]. However, to the best of our knowledge, there were few reports on the development of specific molecular markers of Wheat– *Aegilops geniculate* based on SLAF-seq technology.

Ae. geniculate SY159 showed immunity to resistance powdery mildew at both seedling and adult stages [31, 32]. In order to utilize the *Ae. geniculate* SY159 resistance genes in wheat, distant hybridization, and chromosome manipulation between Chinese Spring and have been backcrossed since 2009s. A series of wheat-*Aegilops geniculate*, including alien chromosome disomic addition line NA0973-5-4-1-2-9-1[32], W19513[33] and disomic substitution line W1698[34] have been characterized by molecular cytogenetic methods. Among wheat-*Aegilops geniculate* derivative lines, alien chromosome $7M^g$ addition line NA0973-5-4-1-2-9-1 and $7M^g$ (7A) substitution line W16998 exhibited high level of resistances to powdery mildew in wheat-growing regions. The objectives of this present study were to (1) constructed and genetic analyzed chromosome $7M^g$ by two genetic populations; (2) determine the resistances of chromosome $7M^g$ using molecular cytogenetic and functional molecular markers methods; (3) developed and utilized more specific markers to verify alien chromosome $7M^g$ resistance using SLAF-Seq technique. The results provide a basis for resistance gene mapping and marker development in future.

Results

Cytological analysis of genetic population

Indoor root tip cytological identification was performed on plant materials CS, SY159, NA0973-5-4-1-2-9-1, W16998, Yuanfeng175 and their constructed F_1 plant and F_2 population. Cytological examination revealed that the number of chromosomes in CS was 42, the number of chromosomes of SY159, NA0973-5-4-1-2-9-1,

W16998 and Yuanfeng175 were 28, 44, 42 and 42, respectively. Among them, the number of F_1 plant chromosomes constructed by NA0973-5-4-1-2-9-1 and Yuanfeng175 was 43 and F_2 plants chromosomes were 42, 43, 44, respectively. The number of F_1 plant chromosomes constructed by W16998 and Yuanfeng175 was 42, and the number of F_2 plants chromosomes were 40, 41, 42, 43 and 44, respectively (Table 1). Thus, the results indicated that the population F_2 produces different types of plants, which is great significance for the selection of resistant materials.

Table 1
Molecular cytological and disease resistance identification of target materials

materials	No. of plants	No. of observed chromosomes	Identification of resistance to powdery mildew
CS	10	42	S
SY159	10	28	R
Yuanfeng175	10	42	S
NA0973-5-4-1-2-9-1	10	44	R
F_1	10	43	R
F_2	43	42	S
	72	43	R
	50	44	R
W16998	10	42	R
F_1	10	42	R
F_2	12	40	S
	30	41	R, S
	59	42	R, S
	32	43	R
	10	44	R

R, resistant; S, susceptible

FISH and FISH-GISH analysis

The chromosomes of the target materials were analyzed by FISH with oligonucleotide probes Oligo-PTa535 and Oligo-pSc119.2, and the chromosomes were compared with CS [35] standard karyotype map (Fig. 1). (Fig. 1A) FISH of CS. (Fig. 1B) FISH of Yuanfeng175. (Fig. 1C) FISH of NA0973-5-4-1-2-9-1. (Fig. 1D) GISH of NA0973-5-4-1-2-9-1 in the same cell. (Fig. 1E-H) NA0973-5-4-1-2-9-1 and Yuanfeng175 constructed the FISH

of F_1, F_2. From the above FISH karyotype map, it was found that there was no variation in chromosome 1A (Fig. 1). F_1 plant has 43 chromosomes, of which only one was exogenous (Fig. 1E). In F_2 plants, the chromosome strips of 42, 43 and 44 were formed. The number of alien chromosomes added was zero, one and two, respectively. (Fig. 1F-H). FISH of W16998. (Fig. 2A) W16998(Fig. 2B) and Yuanfeng175 constructed F_1 FISH. W16998 and Yuanfeng175 constructed F_2 plants FISH, in which the chromosome number was 40 (without alien chromosome), 41 (containing one alien chromosome and no alien chromosome), 42 (containing two alien chromosomes, one alien chromosome and without alien chromosome), 43 (containing two alien chromosomes and one alien chromosome), 44 (containing two alien chromosomes) (Fig. 2C-2L). In summary, Figs. 1 and 2 showed that alien chromosomes could be stably inherited and could produce different gamete types, which was great significance in wheat breeding.

Identification of powdery mildew resistance

After the plants in A and B pots were inoculated with powdery mildew E09 and the resistance to powdery mildew was identified when they were fully infected. The results demonstrated that CS, Yuanfeng175 and susceptible control Shaanyou225 had 4 grades of disease. SY159 showed grade 0 and immunity. NA0973-5-4-1-2-9-1 showed grade 0, high resistance. NA0973-5-4-1-2-9-1×Yuanfeng175 F_1 plants showed high resistance. Three genotypes in NA0973-5-4-1-2-9-1 × Yuanfeng175 F_2 population, with 42 (high susceptibility), 43 (high resistance) and 44 (high resistance) chromosomes respectively. Among 165 F_2 plants, 122 strains were resistant and 43 strains were susceptible. The ratio of resistance to resistance was about 2.8 (Figure. 3A, Table 1). W16998 showed grade 0, high resistance. W16998×Yuanfeng175 F_1 plant showed high resistance, Nine genotypes in W16998×Yuanfeng175 F_2 population, with 40 (high sensitivity), 41 (high resistance), 41 (high resistance), 42 (high resistance), 42 (high resistance), 42 (high susceptibility) 43 (high resistance), 43 (high resistance), and 44 (high resistance) chromosomes respectively. Among 143 F_2 plants, 100 strains were resistant and 43 strains were susceptible. The ratio of resistance to resistance is about 2.3 (Fig. 3B, Table 1). To sum up, genetic analysis indicated preliminarily that concluded that the alien chromosome carries the dominant gene of resistance to powdery mildew and could be stably inherited in the population.

Functional molecular marker analysis

To determine the homologous group relationship of the target materials, DNA was extracted from fresh leaves of the target materials by a modified CTAB method[36]. Then, EST-STS molecular markers were used for analysis, and the bands of heterologous chromosomes with CS, SY159, NA0973-5-4-1-2-9-1, W16998, Yuanfeng175, NA0973-5-4-1-2-9-1×Yuanfeng175 F_1 plant, F_2 population and W16998×Yuanfeng175 F_1 plant, F_2 population that were compared and analyzed. The results showed that the primer *BE637663* (Table 2) could amplify chromosome specific bands and correspond to disease resistance (specific bands appeared in disease-resistant materials and no specificity appeared in susceptible materials) (Fig. 3C-D). It was further proved that chromosome $7M^g$ contained disease resistance gene and could be inherited stably. It provides technical support for genetic improvement of wheat by making better use of excellent genes in alien chromosomes.

Specific-locus amplified fragment sequencing analysis

The SLAF-seq data comprised a total of 4,733,453, 8,752,434, 8,627,924 and 6,977,175 raw reads for CS, SY159, NA0973-5-4-1-2-9-1 and W16998, respectively. The average Q30 of sequencing was 94.15%, and the average GC content was 48.66%. After filtering out low-depth data, the final numbers of SLAF-seq reads were 314,571, 193,701, 388,039 and 362,377 for CS, SY159, NA0973-5-4-1-2-9-1 and W16998, respectively, and the average sequencing depth of the tags was 74.11x. Using the Burrows–Wheeler Alignment (BWA) tool, NA0973-5-4-1-2-9-1 2391 reads were observed to show 50% similarity to the CS wheat reference genome (IWGSC-RefSeqv1.0). Among them reads, there were 961 reads with more than 90% similarity to SY159. In addition, it was found that the similarity between W16998 3980 reads and CS wheat reference genome (IWGSC-RefSeqv1.0) was more than 50%. Among them reads, there were 782 reads with more than 90% similarity to SY159. Finally, the reserved reads of NA0973-5-4-1-2-9-1 and W16998 were compared and analyzed, and the 339 common reads were considered to be specific fragments of chromosomes $7M^g$. A total of 121 primers were designed according to 121 the randomly selected fragments to amplify CS, SY159, NA0973-5-4-1-2-9-1, and W16998 (Table 2, part of the data). Among them, 84 markers showed chromosome $7M^g$ specific bands, with a success rate of 69.4% (Fig. 4). Finally, 84 sequences of chromosome $7M^g$ were obtained.

Table 2

Expressed sequence tag-sequence-tagged site (EST–STS) and special SLAF-seq markers list 7M[g].

Marker	Type	Primer (5′-3′)	Location	Geltype/Restrictionenzyme	Tm ∘C/t (h)
BE637663	EST-SSR	F: ACTGTTGCTTCGCTCCAAGT	7AL 7BL 7DL	8% non-denaturing	60
		R: GTTCCATTTCCGATGTGCTC		polyacrylamide gel 8% non-denaturing/-	
Marker7	SLAF-seq	F: GCTACACCGAACGACAATCA	7M[g]	2% agarose gel	58
		R: AGGTGTCCGCTAAGGATTGA			
Marker9	SLAF-seq	F: CAGACGAGCTTGACTGCTTG	7M[g]	2% agarose gel	58
		R: AATTTGTGCCGATTCAAAGG			
Marker65	SLAF-seq	F: GCTGCAGCAAAGCATTTACA	7M[g]	2% agarose gel	58
		R: AGCACGTCCAAAAGAGGCTA			
Marker945	SLAF-seq	F: CTTCCGAGCTCCGGTAAGAT	7M[g]	2% agarose gel	58
		R: TGATGTGGTGATCCTTTCCA			
Marker1661	SLAF-seq	F: TTGGCATACAAGCCAACTCA	7M[g]	2% agarose gel	58
		R: AGAGTGCTCTGGTCGGAAAG			
Marker2024	SLAF-seq	F: CTTTGGTCCCTTCTTTGGGT	7M[g]	2% agarose gel	58
		R: GCTTAATAGATGCCGAAGCG			
Marker887	SLAF-seq	F: GCATGGGTCCTGTCGACTAT	7M[g]	2% agarose gel	58
		R: TCAGTGGCTGAAGGTCAGTG			
Marker2104	SLAF-seq	F: GCCCAAGGATGAGATGCTAA	7M[g]	2% agarose gel	58
		R: AGAAGAGGGGAAACTCGACC			

Marker	Type	Primer (5′-3′)	Location	Geltype/Restrictionenzyme	Tm ∘C/t (h)
Marker390	SLAF-seq	F: GGAAGTTTACCGAAGATGGC	$7M^g$	2% agarose gel	58
		R: GGGAGCTAAAGAAAGCCGAT			

Marker9, *Marker1661*, *Marker2024* and *Marker390* were randomly selected from the newly developed target chromosome SLAF-seq markers and showed high resistance to CS, SY159, W16998, Yuanfeng175 and W16998×Yuanfeng175 F_1 plants. There were nine genotypes in W16998×Yuanfeng175 F_2 population (Fig. 2), which were 40 (highly susceptible), 41 (highly resistant), 41 (highly resistant), 42 (highly resistant), 42 (highly resistant), 42 (highly susceptible), 43 (highly resistant), 43 (highly resistant) and 44 (highly resistant), respectively. NA0973-5-4-1-2-9-1×Yuanfeng175 F_1 plants showed high resistance. There were three genotypes in NA0973-5-4-1-2-9-1×Yuanfeng175 F_2 population, which were 42 (high susceptibility), 43 (high resistance) and 44 (high resistance), respectively. There were no specific bands in susceptible plants and specific bands in resistant plants, which proves that a number of specific markers on chromosome $7M^g$ have been successfully developed. These specific molecular markers could help us further study and opens up opportunities to develop genomic resources for wild wheat to facilitate crop improvement (Figure. 5).

(M) DL2000 (2kb DNA ladder (1) CS; (2) SY159; (3) W16998; (4) Yuanfeng175; (5) NA0973-5-4-1-2-9-1×Yuanfeng175 F_1 plant; (6–14) Nine genotypes in W16998×Yuanfeng175 F_2 population; (6) F_2 plant (2n = 40, miss two chromosome 7A); (7) F_2 plant (2n = 41, add one alien chromosome and miss two chromosome 7A); (8) F_2 plant (2n = 41, miss one chromosome 7A); (9) F_2 plant (2n = 42, adding two alien chromosome to replace one chromosome 7A); (10) F_2 plant (2n = 42, adding one alien chromosome to replace one chromosome 7A); (11) F_2 plant (2n = 42, without alien chromosome); (12) F_2 plant (2n = 43, add two alien chromosomes and miss one chromosome 7A); (13) F_2 plant (2n = 43, add one alien chromosome); (14) F_2 plant (2n = 44, add two alien chromosomes); (15) NA0973-5-4-1-2-9-1; (16) NA0973-5-4-1-2-9-1×Yuanfeng175 F_1 plant; (17–19) Three genotypes in NA0973-5-4-1-2-9-1×Yuanfeng175 F_2 population; (17) F_2 plant (2n = 42, without alien chromosome); (18) F_2 plant (2n = 43, add one alien chromosome); (19) F_2 plant (2n = 44, add two alien chromosomes); The SLAF-seq markers amplification results with *Marker9*, *Marker1661*, *Marker2024*, *Marker390*. The red arrows indicate the $7M^g$ specific bands.

Discussion

With the continuous development of breeding technology, the newly bred varieties in the market have excellent performance in yield and quality, which made the proportion of resistance genes in the generation of newly bred varieties getting higher and higher. But, the excellent genes of the corresponding farm species have been completely compressed, and even some have disappeared. Breeding new varieties, although harmful genes have been eliminated, beneficial genes have also been eliminated invisibly, resulting in a narrower genetic base of wheat itself [7, 37, 38]. Due to the loss of resistance genes in wheat, the discovery

of new resistance genes from wild related species of wheat has also become the focus of breeders. The resistance genes of wheat powdery mildew were derived from common wheat and its wild relatives [7, 16, 39]. The wild related genera of wheat are rich and diverse. They reproduce under natural conditions and preserve a large number of original genes, which provides a large number of genetic resources for the improvement of wheat genetics and breeding. Mining excellent genes from wild related species of wheat was of great significance in broadening wheat genetic resources, enriching genetic diversity of wheat varieties and promoting the breeding of new varieties. [40]. Wheat distant hybridization is a long-standing and increasingly important method to improve the genetic diversity of common wheat by introducing beneficial genes from related species of wheat[22, 41]. Using distant hybridization and conventional breeding, Wang et al. have introduced *Fhb7* into diverse wheat backgrounds for wheat improvement in wheat and was validated by genomic in situ hybridization [42].

Ae. geniculata contains a large number of excellent genes which could be used to improve wheat genetics and breeding. These genes were distributed on different chromosomes of *Ae. geniculate* [31]. It has been a hot spot in wheat distant hybridization research to make use of the excellent traits shown by these genes. *Aegilops* and *Triticum* are most closely related, and there is a strong genetic similarity between them [43]. Thus, excellent genes from *Ae. geniculata* could be easily introduced into wheat by hybridization. Previous studies have shown that three disease resistance genes (*Pm29*, *Yr40*, *Lr57*, *Sr53*) have been found in *Ae. geniculate* [26, 27, 44].

In the present experiment, in this study, cytological identification and analysis showed that NA0973-5-4-1-2-9-1 and W16998 could obtain the number of chromosomes of different types by constructing the F_1 plant and F_2 population, which laid a more abundant material foundation for the utilization of their excellent trait genes (Table 1). FISH has become a useful tool for detecting chromosomal exogenous sources [28]. Tang et al. have developed some oligonucleotide probes based on new tandem repeat sequences, which were of great significance for the identification of wheat chromosomes or specific chromosome segments [35]. The GISH technology described by Yang et al. was used with minor modifications [45]. Through FISH and GISH techniques, it was preliminarily determined that NA0973-5-4-1-2-9-1 and W16998 contained the same pair of alien chromosomes $7M^g$ (Figs. 1 and 2). At the same time, by comparing the karyotypes of the population constructed by NA0973-5-4-1-2-9-1 and Yuanfeng175, the number of chromosomes was added to the F_2 population with the number of chromosomes zero, one and two, respectively (Fig. 1F-H). However, nine karyotypes were found in the F_2 population constructed by W16998 and Yuanfeng175 (Fig. 2C-L). It laid a foundation for the creation of small fragment translocation lines and osmosis lines which could be directly used in production and the mining of these excellent genes. The results of powdery mildew in parents, F_1 and F_2 generations showed that the response of plants containing chromosomes $7M^g$ components of SY159 to race E09 was consistent with that of parents NA0973-5-4-1-2-9-1 and W16998. Compared with the standard CS karyotype, it was found that 1A had no variation in Fig. 1, while the green signal appeared in the short arm of 1A in Fig. 2. Besides, the ratio of resistant plants to susceptible plants in their population given with Mendelian genetic law and FISH karyotype, we found that there was a variation in chromosome 1A and there was no alien chromosome (Fig. 2E-F). This type was obviously susceptible, which fully indicated that

the disease resistance was only related to alien chromosome 7M^{g}, indicating that the population was highly resistant to powdery mildew whether it contained one or two exogenous bands (Fig. 3).

EST-SSR has an accurate and reliable function in identifying the homologous relationship between alien chromosomes and wheat chromosomes. Wang et al. successfully identified rye 1RS chromosomes by EST-SSR markers [46]. At present, rye genetic maps have been successfully constructed by using different marker techniques [40]. Using the markers on these maps, the components of rye chromatin under the background of wheat could be identified successfully. In this study, we have used one functional molecular marker to quickly detect that the disease resistance of the target plant has the same specificity as that of *Ae. geniculata* SY159. The results showed that the specific differences of markers corresponded to their karyotype, alien chromosomes and disease resistance. The result depended on the existence of alien chromosome 7M^{g}. It was preliminarily confirmed that there was a dominant powdery mildew resistance gene in the target material.

It was difficult to develop chromosome specific markers for related species of wheat by conventional methods. Wu et al. screened 2 RAPD markers specific to *Agropyron cristatum* from 520 RAPD primers [47]. In recent years, with the development of molecular marker technology, a variety of molecular markers have been developed to detect the existence of exogenous genetic material under the background of wheat, especially the molecular markers closely interlocked with functional genes could be used to assist breeding. SLAF-seq a high-throughput sequencing technique for screening specific length DNA fragments by constructing a SLAF-seq library, obtaining a large number of sequences by high-throughput sequencing technology, analyzing and comparing data with software, obtaining a large amount of sequence information, and then developing specific molecular markers according to its sequences. Specific molecular markers of *Th. elongatum* were developed by Chen et al. with efficiencies up to 65.9% [48]. Song et al. designed the 242 markers showed *Ae. biuncialis* specific bands with a success rate up to 40.33% [49]. Sixty-one pairs of molecular markers were screened about *Ae. geniculata* by Wang et al, with an effective rate of 47.66% [33]. In the study, 339 fragments specific for the 7M^{g} chromosome were obtained using SLAF-seq technology based on 121 randomly selected fragments. 84 pairs of molecular markers were screened with an effective rate of 69.4%. In the improvement of wheat genetics and breeding, addition lines, substitution lines and translocation lines, breeders prefer translocation lines because of its low genetic resistance and could be directly used in small quantities [13, 50]. To develop small-segment translocation or introgression lines with powdery mildew genes. Methodology is available to induce target chromosome variation by ^{60}Coγ radiation [51]. At present, this laboratory has obtained a number of high-generation irradiated materials containing the alien chromosome 7M^{g}. In the later stage, the specific markers developed by SLAF-seq technology could be used to quickly identify the excellent genes on the chromosome. SLAF-seq could be used not only to trace the existence of alien chromosomes under the background of wheat, but also to fine map the genes that control excellent traits on alien chromosomes [52].

Conclusions

In summary, the powdery mildew resistance gene in this paper was a dominant gene with strong resistance. The new trait genes could be inherited stably, thus enriching the available genetic resources in *Ae.*

geniculata. Moreover, a batch of markers which could detect the 7M^g chromosome of *Ae. geniculata* were developed by using the tag sequence obtained by simplified genome sequencing for the first time. This laid a foundation for the further creation of translocation lines or osmosis lines containing powdery mildew resistance genes that could be used in wheat improvement.

Materials And Methods

Plant materials

The wheat – *Ae. geniculata* 7M^g disomic addition line NA0973-5-4-1-2-9-1 and 7M^g (7A) alien disomic substitution line W16998 were identified by Wang et al [32, 34]. NA0973-5-4-1-2-9-1 and W16998 were derived from a cross between CS and *Ae. geniculate* SY159. They were crossed with a powdery mildew susceptible common wheat Yuanfeng175, respectively. Then, F_1 and F_2 generations were constructed by strictly self-crossed, respectively. Common wheat Shaanyou 225 was used as the susceptible control for assessment of powdery mildew resistance. The *Aegilops geniculata* SY159 (Unit preservation number Y359) collected in Syria in 1992, was kindly provided by Professor Lihui Li and Xinming Yang (the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. Beijing, 100081, P. R. China). The common wheat Yuanfeng175 was authorized varieties from Shaanxi province in 2005 (accession 2005006), common wheat Shaanyou 225 was authorized varieties from Shaanxi province in 1993 (accession 1993257), common wheat 'Chinese Spring' (CS) (AABBDD, 2n = 42) is a local variety originating from Chengdu, Sichuan, China. All the materials were maintained at Wheat Distant Hybridization and Molecular Chromosome Engineering Laboratory, the College of Agronomy, Northwest A&F University. Yangling, 712100, P. R. China.

Cytological analysis of genetic population

Soak the target seeds in a petri dish with a filter paper (the water in the dish should cover the seeds) and place them in a 23°C incubator in the dark for about 24 hours. Then, pour out the water from the petri dish, arrange it neatly, and continue to put it in the incubator, while making sure the petri dish stays moist throughout the rooting process. In accordance with the method of Yang et al [53], when the roots grew to 2–3 cm, the roots were excised and placed in a previously perforated centrifuge tube and treated with nitrous oxide (N_2O) for ~ 2h. Then, it was immobilized in 95% acetic acid for 30 minutes and stored in 70% ethanol for later use. The root tip was treated with 1% pectinase and 2% cellulose in a water bath at 37°C for ~ 1h, and then made into white tablets. At last, the chromosomes number of root tip cells were observed by an Olympus BX43 Microscope (Janpan) equipped with a Photometrics SenSys CCD camera and recorded by photography.

FISH and FISH-GISH analysis

Using the Oligonucleotide probes Oligo-PTa535(red) and Oligonucleotide probes Oligo-pSc119.2 (green) as Fluorescence in situ hybridization, which were compared with existing CS karyotypes to determine the alien chromosome profile [35]. Then, the white tablet after fluorescence *in situ hybridization* was soaked and dried in alcohol and exposed to dry, and then genomic *in situ hybridization* (GISH) was carried out. GISH would extract and purify SY159 DNA as a probe, different concentrations of Chinese spring DNA mixed after

blocking, according to the ratio of 1:300, at a suitable temperature of 56°C for hybridization. Finally, the chromosome signals were observed under a phase contrast microscope to determine whether there was a foreign source. Combining the results of these two techniques could further determine whether the chromosomes of the derived materials carry alien genes. The chromosomes were stained with the blue-fluorescent DNA stain 4',6-diamidino-2-phenylindole (DAPI) [32]. Select and analyze the karyotype image with better signal display (Olympus bx-53).

Assessment of powdery mildew resistance

The degree of resistance to powdery mildew was assessed at the seedling stage in artificial climate incubator. The populations constructed by NA0973-5-4-1-2-9-1 and W16998 were planted in pots A and B of 28 x 52 cm, respectively. In A pot, the first to sixth rows were planted with Shaanyou225, CS, SY159, material NA0973-5-4-1-2-9-1, Yuanfeng175, F_1 plant, each with 10 plants. 165 F_2 plants were planted in the remaining space. Similarly, in the B pots, the first to sixth rows were planted with Shaanyou225, CS, SY159, W16998 and Yuanfeng175, F_1 plant, respectively, with 10 plants. 143 F_2 plants were planted in the remaining space. Among them, Shaanyou225 was the susceptible control. According to the method stated in the study by Zhao et al.[54], the plants were inoculated with *Bgt* physiological race E09 by artificial shaking when conidiospores obtained from the susceptible cultivar Shaanyou225 at the two-leaf stage. After ~2 weeks, when the symptoms of the disease were evident in the plant, the level of infection was described as 0–4, 0 indicates immunity, 0; indicates near immunity, 1 indicates high resistance, 2 indicates moderate resistance, 3 indicates moderate sensitivity, and 4 indicates high sensitivity [55].

Molecular marker analysis

According to the previous studies on the chromosomes of *Ae. geniculata* SY159 by Wang et al. [32, 34], which have selected only one EST-STS (Expressed sequence tag-sequence-tagged site) functional molecular marker from among the PCR Primers for Grain Genes database (http://wheat.pw.usda.gov/SNP/new/pcr_primers.shtml) [56] to study the two genetic populations constructed in this experiment. DNA Amplification and later data processing were performed using a modified Zhu et al. method [57]. PCR products were subjected to polyacrylamide gel electrophoresis and stained with 1% $AgNO_3$ solution. Finally, the difference bands were observed and analyzed.

Specific-locus amplified fragment sequencing analysis

In recent years, in the field of molecular markers, the development of specific heterogeneous chromosome markers is of great significance in breeding [48]. Then, SLAF-seq of NA0973-5-4-1-2-9-1 and W16998 were performed with some modification by the Beijing Biomarker Technologies Corporation [49]. To improve the specificity and efficiency of the marker, firstly, the SLAF-seq of NA0973-5-4-1-2-9-1 and W16998 was compared with the CS sequences (http://www.wheatgenome.org/News/Latest-news/IWGSC-Reference-Sequencev1.0-browser-now-available-at-URGI), and the sequences with similarity less than 50% were reserved. Secondly, compared the preserved sequences with SY159 sequences respectively, the sequences with more than 90% similarity were preserved. Finally, the common sequence was designed as a specific primer [30, 33, 48]. The PCR amplification of modified was performed following the method previously

described by Luan et al. [58]. The annealing temperature based on SLAF-seq markers were 59 ◦C. The amplified products were electrophoretic on a preprepared 2% agarose gel. After the end of electrophoresis, the polymorphism was observed and photographed on the gel imager. The specific primers designed in the experiment were synthesized by the Beijing Aoke Ding Sheng Biotechnology Co., Ltd. (Beijing, China).

Declarations

Acknowledgements

None.

Authors' Contributions

Y.W. (Yongfu Wang), Y.W. (Yajuan Wang) and W.J. designed the study, analyzed the data and Y.W. (Yongfu Wang) wrote the article. Y.W. (Yajuan Wang) and Y.W. (Yongfu Wang) contributed to the development of material. J.F. and X.X contributed to molecular cytological analysis. X.F contributed to the SLAF-seq data analysis. H.Z, and C.C contributed to powdery mildew resistance evaluation. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets used and materials during the current study are available from the corresponding author on reasonable request. The data sets were deposited in the China National Center for Bioinformation (CNCB) database under accession number PRJCA009052.

Ethics approval and consent to participate

Aegilops geniculata Roth was wild plant in this study. All plants used here were permitted and obtained from Northwest A&F University. All related plant materials are available and comply Wild Plants Protection Regulation of China.

Competing interests

The authors declare no conflict of interest.

Consent for publication

Not applicable.

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Figures

Figure 1

The chromosome karyotype of the target plant was analyzed by FISH and continuous GISH. FISH uses Oligo-pSc119.2 (green) and Oligo-pTa535 (red) as probes. SY159 genomic DNA was mixed with CS and used as a probe for sequence GISH. The red arrow indicates the alien chromosome introduced into SY159. The structural variation of the chromosome was indicated by the white arrow chromosome. (A) FISH of CS. (B) Analysis of Yuanfeng175 by FISH. (C) The FISH of NA0973-5-4-1-2-9-1. (D) GISH of NA0973-5-4-1-2-9-1 in the same cell. (E-H) NA0973-5-4-1-2-9-1 and Yuanfeng175 constructed F_1, F_2 FISH. (E) F_1 plant (2n=43, add one alien chromosome). (F) F_2 plant (2n=42, without alien chromosome). (G) F_2 plant (2n=43, add one alien chromosome). (H) F_2 plant (2n=44, add two alien chromosomes). Chromosomes were counterstained by DAPI (blue). Bar indicates 10 μm.

Figure 2

The karyotype of the target plant was analyzed by FISH. FISH uses Oligo-pSc119.2 (green) and Oligo-pTa535 (red) as probes. The red arrow indicates the alien chromosome introduced into SY159. The structural variation of the chromosome was indicated by the white arrow chromosome. (A) FISH of W16998. (B) The FISH of F_1. (C-L) F_2 constructed by W16998 and Yuanfeng175. (C) F_2 plant (2n=40, miss two chromosome 7A). (D) F_2 plant (2n=41, add one alien chromosome and miss two chromosome 7A). (E) F_2 plant (2n=41, miss one chromosome 7A). (F) F_2 plant (2n=41, miss one chromosome 7A). (G) F_2 plant (2n=42, adding two alien chromosome to replace one chromosome 7A). (H) F_2 plant (2n=42, adding one alien chromosome to replace one chromosome 7A). (I) F_2 plant (2n=42, without alien chromosome). (J) F_2 plant (2n=43, add two alien chromosomes and miss one chromosome 7A). (K) F_2 plant (2n=43, add one alien chromosome). (L) F_2 plant (2n=44, add two alien chromosomes). Chromosomes were counterstained by DAPI (blue). Bar indicates 10 μm.

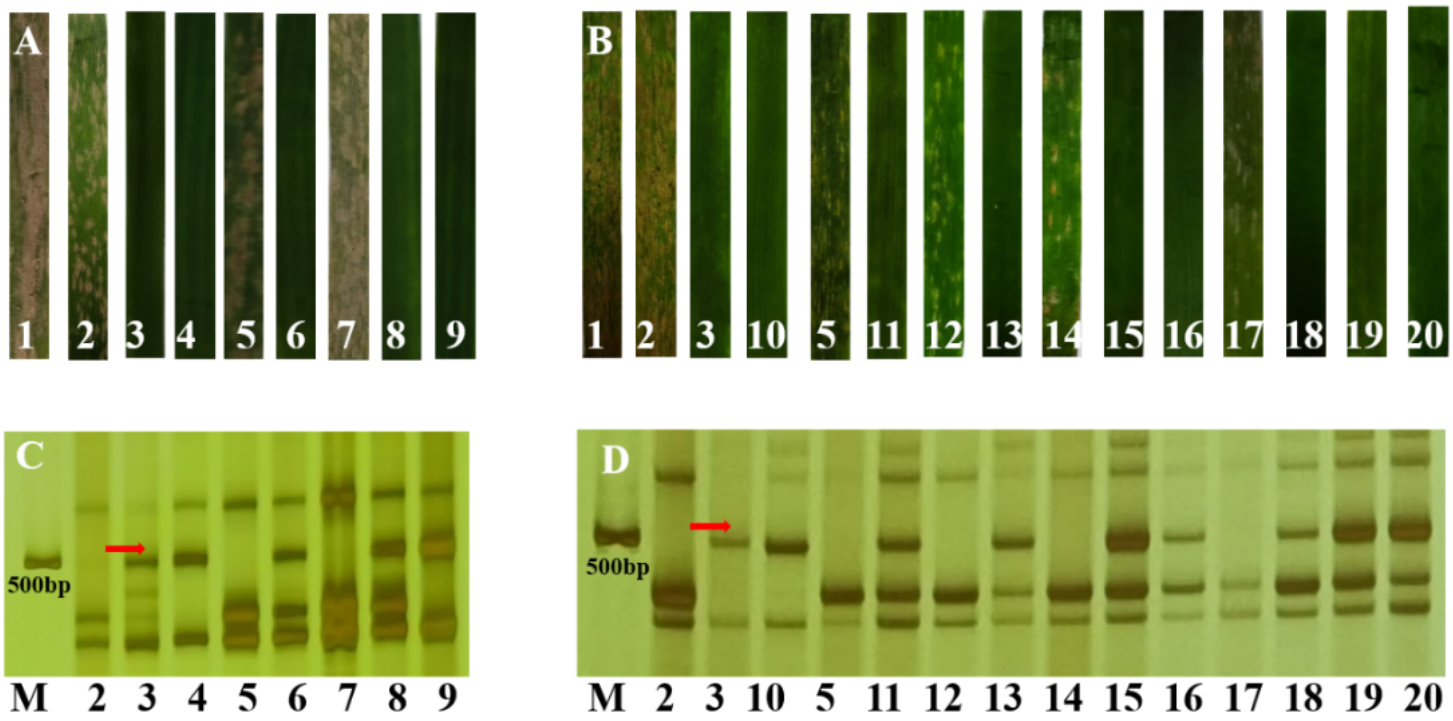

Figure 3

Powdery mildew reaction (Figure A-B) and EST–STS functional molecular marker analysis of the target material (Figure C-D). (1) Shaanyou225; (2) CS; (3) SY159; (4) NA0973-5-4-1-2-9-1; (5) Yuanfeng175; (6) NA0973-5-4-1-2-9-1×Yuanfeng175 F_1 plant; (7-9) Three genotypes in NA0973-5-4-1-2-9-1×Yuanfeng175 F_2 population;(7) F_2 plant (2n=42, without alien chromosome); (8) F_2 plant (2n=43, add one alien chromosome); (9) F_2 plant (2n=44, add two alien chromosomes); (10) W16998; (11) W16998×Yuanfeng175 F_1 plant; (12-20) Nine genotypes in W16998×Yuanfeng175 F_2 population, (12) F_2 plant (2n=40, miss two chromosome 7A). (13) F_2 plant (2n=41, add one alien chromosome and miss two chromosome 7A). (14) F_2 plant (2n=41, miss one chromosome 7A). (15) F_2 plant (2n=42, adding two alien chromosome to replace one chromosome 7A). (16) F_2 plant (2n=42, adding one alien chromosome to replace one chromosome 7A). (17) F_2 plant (2n=42, without alien chromosome). (18) F_2 plant (2n=43, add two alien chromosomes and miss one chromosome 7A). (19) F_2 plant (2n=43, add one alien chromosome). (20) F_2 plant (2n=44, add two alien chromosomes). (M) DL2000 (2kb DNA ladder); (Fig. C-D) The EST–STS markers amplification results with *BE637663*. The red arrows state the specific bands.

Functional molecular marker analysis

Figure 4

Amplification patterns of Aegilops accessions using NA0973-5-4-1-2-9-1 and W16998. SLAF markers *Marker7*, *Marker9*, *Marker65*, *Marker945*, *Marker1661*, *Marker2024*, *Marker887*, Marker2104 and Marker390.

(M) DL2000 (2 kb DNA ladder). (1). CS. (2) SY159. (3). NA0973-5-4-1-2-9-1. (4). W16998. The red arrows indicate the specific bands.

Figure 5

Molecular marker development and PCR amplification in target material (Figure A-D).

(M) DL2000 (2kb DNA ladder (1) CS; (2) SY159; (3) W16998; (4) Yuanfeng175; (5) NA0973-5-4-1-2-9-1×Yuanfeng175 F_1 plant; (6-14) Nine genotypes in W16998×Yuanfeng175 F_2 population; (6) F_2 plant (2n=40, miss two chromosome 7A); (7) F_2 plant (2n=41, add one alien chromosome and miss two chromosome 7A); (8) F_2 plant (2n=41, miss one chromosome 7A); (9) F_2 plant (2n=42, adding two alien chromosome to replace one chromosome 7A); (10) F_2 plant (2n=42, adding one alien chromosome to replace one chromosome 7A); (11) F_2 plant (2n=42, without alien chromosome); (12) F_2 plant (2n=43, add two alien chromosomes and miss one chromosome 7A); (13) F_2 plant (2n=43, add one alien chromosome); (14) F_2 plant (2n=44, add two alien chromosomes); (15) NA0973-5-4-1-2-9-1; (16) NA0973-5-4-1-2-9-1×Yuanfeng175 F_1 plant; (17-19) Three genotypes in NA0973-5-4-1-2-9-1×Yuanfeng175 F_2 population; (17) F_2 plant (2n=42, without alien chromosome); (18) F_2 plant (2n=43, add one alien chromosome); (19) F_2 plant (2n=44, add two alien chromosomes); The SLAF-seq markers amplification results with *Marker9*, *Marker1661*, *Marker2024*, *Marker390*. The red arrows indicate the $7M^g$ specific bands.

Supplementary Files

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