Molecular cytogenetic identification of a wheat (*Triticum aestivum*)-American dune grass (*Leymus mollis*) translocation line resistant to stripe rust

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ABSTRACT. *Leymus mollis*, a perennial allotetraploid (2n = 4x = 28), known as American dune grass, is a wild relative of wheat that could be useful for cultivar improvement. Shannong0096, developed from interspecific hybridization between common wheat cv. Yannong15 and *L. mollis*, was analyzed with cytological procedures, genomic in situ hybridization, stripe-rust resistance screening and molecular marker analysis. We found that Shannong0096 has 42 chromosomes in the root-tip cells at mitotic metaphase and 21 bivalents in the pollen mother cells at meiotic metaphase I, demonstrating cytogenetic stability. Genomic in situ hybridization probed with total genomic DNA from *L. mollis* gave strong hybridization signals in the distal
Identification of a wheat-\textit{Leymus mollis} translocation

A single dominant \textit{Yr} gene, derived from \textit{L. mollis} and temporarily designated as \textit{YrSn0096}, was found on the long arm of chromosome 4A of Shannong0096. \textit{YrSn0096} should be a novel \textit{Yr} gene because none of the previously reported \textit{Yr} genes on chromosome 4A are related to \textit{L. mollis}. This gene was found to be closely linked to the loci \textit{Xbarc236} and \textit{Xksum134} with genetic distances of 5.0 and 4.8 cM, respectively. Based on data from 267 F\textsubscript{2} plants of Yannong15/Huixianhong, the linkage map of \textit{YrSn0096}, using the two molecular markers, was established in the order \textit{Xbarc236-\textit{YrSn0096}-Xksum134}. Shannong0096 appeared to be a unique wheat-\textit{L. mollis} translocation with cryptic alien introgression. Cytogenetic stability, a high level of stripe-rust resistance, the common wheat background, and other positive agronomic traits make it a desirable donor for introducing novel alien resistance genes in wheat breeding programs, with the advantage of molecular markers that can be used to confirm introgression.

\textbf{Key words:} Genomic \textit{in situ} hybridization; \textit{Leymus mollis}; Stripe rust; Translocation

\section*{INTRODUCTION}

As the second most frequently planted crop, wheat (\textit{Triticum aestivum} L.) plays an important role in food security for the world’s population. More than 75\% of the world’s population is estimated to consume wheat as part of the daily diet (Afzal et al., 2008). To meet the needs of the growing world population, the demand for wheat is expected to grow faster than that for any other major agricultural crop (Dreisigacher, 2004). However, wheat production is hampered by many diseases.

Stripe rust (syn. yellow rust), caused by \textit{Puccinia striiformis} Westend. \textit{f. sp tritici} Eriks, is one of the most devastating fungal diseases of wheat in many cool and temperate regions (Wan et al., 2007). In recent years, this disease has become increasingly important in some wheat-growing areas, where it had previously been absent or not regularly destructive (Zhang et al., 2009). Stripe rust inflicts not only yield losses - ranging from 10 to 70\% and, in some cases, up to 100\% - but also quality downgrades (Chen, 2005). The application of fungicides has been a preferred method for the rapid and effective control of stripe rust. It can also cause large expenditures, contribute to serious environmental and health problems, and result in the selection of fungicide-resistant strains of the pathogen, however (Chen, 2005; Cao et al., 2008). Breeding resistant cultivars has been considered the most effective, economical, and environmentally acceptable approach to stripe rust control (Chen, 2005; Cao et al., 2008; Sui et al., 2009; Hu et al., 2011). To date, more than 50 officially named \textit{Yr} genes at 49 loci (\textit{Yr1-Yr49}) and some temporarily designated genes have been documented (the Komugi Wheat Genetic Resources Database, seeing the reference a for the URL [http://www.shigen.nig.ac.jp/wheat/komugi/genes/symbolListPageAction.do?page=-1]). Most of these genes are race specific, and virulences to them have been detected in various parts of the world (Li et al., 2011). Consequently, many \textit{Yr} genes have lost their resistance to the fungus (Kang et al., 2010), necessitating
a constant search for and transfer of new and effective stripe rust resistance genes.

One reality is that the replacement of highly variable land races by high-yielding, pure-line varieties in many parts of the world has narrowed the genetic base for disease resistance in the wheat gene pool (Kuraparthy et al., 2007a). Fortunately, relatives of wheat have been found to be invaluable sources of disease-resistance genes (Jiang et al., 1994; Friebe et al., 1996). So far, an increasing number of stripe rust resistance genes originating in the wild relatives of wheat have been identified, such as Yr9 from Secale cereale (the Komugi Wheat Genetic Resources Database, seeing the reference b for the URL [http://www.shigen.nig.ac.jp/wheat/komugi/genes/symbolDetailAction.do?geneid=1637]), Yr28 from Aegilops tauschii (Singh et al., 2000), and Yr40 from Aegilops geniculata (Kuraparthy et al., 2007a).

Leymus mollis (Trin.) Hara, a perennial allotetraploid (2n = 4x = 28), is an invaluable wild relative for wheat improvement because of its many superior agronomic characters such as disease resistance, saline-alkali tolerance, exceptionally large spikes with numerous florets, and vigorous growth (Wang et al., 2000; Kishii et al., 2003). The distant relationship presents obstacles to obtaining hybrids between L. mollis and wheat, however. In the 1980s, Fu et al. (1993) acquired the first wheat-L. mollis hybrids through embryo rescue and subsequently created alien chromosome lines, including partial amphidiploids, additions, and substitutions, with varying positive traits derived from L. mollis (Fu et al., 1996, 1997). Few reports are available, however, on wheat-L. mollis translocations, the most valuable germplasms transferring beneficial genes from L. mollis to common wheat. Line Shannong0096, derived from the progeny of a wheat-L. mollis octoploid and bread wheat cv. Yannong15, displays excellent resistance to stripe rust as well as other favorable agronomic characteristics such as numerous tillers and vigorous growth. Herein, we report the cytogenetic identification of Shannong0096 and molecular analysis of its stripe rust resistance.

MATERIAL AND METHODS

Plant materials

Plant materials used in this study included L. mollis, a wheat-L. mollis octoploid, Shannong0096, and wheat varieties Chinese Spring, Yannong15, and Huixianhong. An F2 segregation population was construed on the basis of the cross between Shannong0096 and Huixianhong (highly susceptible to stripe rust). L. mollis and the wheat-L. mollis octoploid were provided by Professor Jie Fu, retired from the Northwest Institution of Botany, Chinese Academy of Sciences, Yangling, China. Shannong0096 was developed from the progeny of Yannong15 and the wheat-L. mollis octoploid at the Agronomy College of Shandong Agricultural University, Tai’an, China. All plant materials were preserved via selfing at the Tai’an Subcenter of the National Wheat Improvement Center, Shandong, China.

Cytogenetic identification

Seeds were germinated at 25°C on moist filter paper on Petri dishes, maintained at 4°C for approximately 24 h, and then returned to 25°C. Roots (1-2 cm) were cut and immersed in ice water for approximately 24 h before fixing in Carnoy’s solution. After fixation, the root tips were stained and flattened in carbol fuchsin, and mitotic chromosomes were observed un-
under a microscope. When plants attained the flag-leaf stage, spikes were sampled, and anthers at metaphase I of meiosis were fixed in Carnoy’s solution, dissociated in 1 M HCl at 60°C for 6-8 min, and flattened in 1% aceticarmine.

For genomic in situ hybridization (GISH), the total genomic DNA from *L. mollis* was labeled with digoxigenin-11-2'-deoxyuridine-5'-triphosphate with the nick translation method and used as a probe. Sheared genomic DNA from Chinese Spring wheat (ABD genomes, 2n = 42) was used as blocking DNA. Detailed procedures of chromosome preparation and hybridization mixture are described elsewhere (Chen et al., 1998). The GISH signals were detected with fluorescein-conjugated anti-digoxigenin antibodies, and the slides were mounted in Vectashield antifade solution containing propidium iodide. Photographs were taken with a fluorescence microscope equipped with a charge-coupled device camera.

**Stripe rust screening**

A mixture of *P. striiformis* races, including Chinese Yellow Rust 29, Chinese Yellow Rust 31, Chinese Yellow Rust 32, Shuyuan11, Shuyuan12, Shuyuan13, and Shuyuan14, was used to evaluate resistance to stripe rust. Shannong0096, Yannong15, *L. mollis*, the wheat-*L. mollis* octoploid, and the *F₂* population of Shannong0096/Huixianhong were planted along with Huixianhong at the Research Farm of Shandong Agricultural University, Tai’an (116°20’~117°59’ E, 35°38’~36°28’ N), Shandong, China. Artificial inoculation was carried out twice with the mixed races at the jointing stage and 7 days later. The infection types (ITs) were classified 21 days after inoculation according to a 0 to 4 scale (Bariana and McIntosh, 1993), on which 0 indicated the presence of no visible symptoms or no signs of infection or necrotic flecks, 1 indicated the presence of necrotic and chlorotic areas with restricted sporulation, 2 indicated the presence of moderate sporulation with necrosis and chlorosis, 3 indicated the presence of sporulation with chlorosis, and 4 indicated the presence of abundant sporulation without chlorosis. Plants with ITs of 0-2 were considered resistant, and those with ITs of 3-4 were deemed susceptible.

**Microsatellite screening and electrophoretic analysis**

Microsatellite primers, designated as either simple sequence repeats (SSRs) or expressed sequence tag-derived SSRs (EST-SSRs), were used to scan the plant materials for useful polymorphisms. SSR primers were synthesized following the sequences published in the GrainGenes database (http://wheat.pw.usda.gov), and the EST-SSR primers were provided by Professor Sishen Li, Agronomy College of Shandong Agricultural University, Tai’an, China. Each 25-μL polymerase chain reaction (PCR) solution contained 2.5 μL 10X PCR buffer, 2.0 μL Mg²⁺ (2.5 mM), 1.5 μL deoxyribonucleotide triphosphates (2.5 nM), 1.0 U *Tag* polymerase, 3.0 μL primer pair (5 μM), and 3.0 μL DNA (30 ng/μL). DNA amplification was carried out in a GeneAmp PCR System 9600 programmed for 4 min at 94°C for pre-denaturation and 35 cycles each consisting of 45 s at 94°C, 30 s at 50°-60°C (depending on the primers), and 1 min at 72°C, followed by a final extension for 10 min at 72°C. The PCR-amplified fragments were separated on a 6% non-denaturing polyacrylamide gel, stained with silver nitrate, and visualized using a Tanon Gis-2010 gel-imaging system.
Data analysis

Chi-square tests were used to evaluate the goodness of fit of observed and expected ratios for stripe rust reactions and molecular markers. The linkage relationship between the markers and the resistance gene was analyzed using the Mapmaker/Exp software, Version 3.0.

RESULTS

Cytological identification of Shannong0096

Chromosome counts and meiotic observation of pollen mother cells in 15 Shannong0096 plants revealed the presence of 42 chromosomes in the root-tip cells (Figure 1A) and 21 bivalents at meiotic metaphase I (Figure 1B) indicative of the cytogenetic stability of Shannong0096. Using digoxigenin-labeled total genomic DNA from *L. mollis* as a probe and Chinese Spring DNA as a blocker, GISH was carried out to detect the alien genetic materials in Shannong0096. In mitotic metaphase cells, two chromosomes were labeled green in the terminal regions and the others were counterstained red (Figure 1C), indicating that Shannong0096 was a translocation line with a small fragment from *L. mollis* translocated to the distal region of a wheat chromosome.

![Figure 1. Cytological patterns of Shannong0096. A. A root-tip cell at mitotic metaphase (2n = 42). B. A pollen mother cell at meiotic metaphase I (2n = 21II). C. A genomic in situ hybridization pattern probed with total genomic DNA from *Leymus mollis*.](image)

Reactions to stripe rust

After inoculation with stripe rust isolates, both the parent Yannong15 and the control Huixianhong were susceptible, whereas Shannong0096, *L. mollis*, and the wheat-*L. mollis* octoploid were immune (Table 1). In addition, all the wheat parents of the wheat-*L. mollis* octoploid-namely, 7182-0-11-1, Dasui78-3, and Zhi763-were susceptible (Zhou et al., 2001). Therefore, the pedigree persuaded us to conclude that YrSn0096 originated from *L. mollis*. 

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Genetic analysis of stripe rust resistance in Shannong0096

To determine the inheritance of the resistance gene(s) in Shannong0096, we developed an F2 population derived from the cross Shannong0096/Huixianhong. After infection by the mixed races of stripe rust, these plants showed apparent symptoms of reactions related to resistance or susceptibility in a resistant:susceptible ratio of 3:1 (Table 2). Thus, stripe rust resistance in Shannong0096 indicated a typical dominant inheritance proved to be controlled by a single gene or locus, which was temporarily designated YrSn0096.

Molecular linkage for YrSn0096

Of the 1261 SSR and EST-SSR primer pairs used for screening, Xbarc236255 and Xksum134245 were identified as potential linkage markers for YrSn0096. For instance, the primer BARC236 generated two special bands (Figure 2): one present in susceptible or resistant parents, designated as Xbarc236250, and the other present only in resistant parents, designated Xbarc236255. The resistant F2 plants amplified Xbarc236255 (homozygous) or both Xbarc236250 and Xbarc236255 (heterozygous), whereas the susceptible bulk and the susceptible F2 individuals amplified only the BARC236250 band.

To obtain genetic data, we further analyzed the genotypes of the loci Xbarc236 and Xksum134 in 267 F2 individuals (Table 3). In the case of Xbarc236, of 199 resistant plants, 76 carried Xbarc236255 (homozygous), 120 carried both Xbarc236250 and Xbarc236255 (heterozygous), and three carried Xbarc236250 (recombinant). The remaining plants were suscep-
tible and carried Xbarc236. Therefore, the F$_2$ genotypes segregated in a 1AA:2Aa:1aa ratio, which confirmed the involvement of a single gene in their inheritance of resistance. The same pattern was observed in the EST-SSR marker Xksum134. Using the Mapmaker software, we found the linkage relationship of Xbarc236 and Xksum134 with YrSn0096 (Figure 3). The two loci Xbarc236 and Xksum134 were genetically associated with YrSn0096, with distances of 5.0 and 4.8 cM, respectively. Based on the published chromosomal location of Xbarc236 (the GrainGenes Database, seeing the reference c for the URL [http://wheat.pw.usda.gov/cgi-bin/graingenes/report.cgi?class=locus;name=Xbarc236-4A]) and Xksum134 (Yu et al., 2004), YrSn0096 was deduced to be located on the long arm of wheat chromosome 4A.

<table>
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<tr>
<th>Markers</th>
<th>Phenotypes</th>
<th>Genotypes</th>
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<th>Expected ratio</th>
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<td>Aa</td>
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R = resistant; S = susceptible.

**Figure 3.** Linkage map of the stripe-rust resistance gene YrSn0096, flanked by two markers on the wheat chromosome 4AL.

**DISCUSSION**

Wild relatives and related species are valuable reservoirs for broadening the genetic variability of common wheat. Generally, the first step for transferring exotic genes is the production of an amphidiploid (or partial amphidiploid) between wheat and the alien species, followed by the production of chromosome addition or substitution lines, and finally, the production of translocation lines. Producing these lines is an enormous and time-consuming chore. Moreover, many derivatives of these wheat-alien species lose the resistance derived from alien species at a high rate (Cao et al., 2008) owing to the cytological instability of alien chromosome segments incorporated into non-homoeologous regions (Nasuda et al., 1998). Shannong0096 was developed directly from the progeny of a wheat-L. mollis octoploid and
Yannong15 wheat, the latter used as the recurrent parent. The present study demonstrates its cytological stability and excellent resistance to stripe rust, suggesting the feasibility of transferring alien genes to wheat with genetic stability directly by crossing and backcrossing.

Genetic information, including inheritance, type, and number of resistance genes, could be used to predict the durability of resistance in breeding lines and commercial cultivars (Sui et al., 2009). In the present study, we identified a stripe rust resistance gene, \( YrSn0096 \), in the wheat-\( L.~mollis \) translocation line Shannong0096. Similar to many reported \( Yr \) genes, \( YrSn0096 \) is controlled by a single, dominant locus. It resists prevailing stripe rust races, including the predominant race Chinese Yellow Rust 32, which caused resistance loss in nearly all Chinese wheat cultivars and, consequently, a large epidemic of the fungus in China in 2002. We localized this gene to the long arm of wheat chromosome 4A using the markers BARC236 and KSUM134. The previously reported \( Yr \) genes \( YrHVII, YrMtn, \) and \( YrND \) are also located on this chromosome (the Komugi Wheat Genetic Resources Database, seeing the reference a for the URL), but none of them is related to \( L.~mollis \). Therefore, \( YrSn0096 \) must be a novel \( Yr \) gene. Compared to the incorporation of other exotic sources, incorporating the high-resistance gene \( YrSn0096 \) into different wheat cultivars should be relatively easy owing to the common wheat background and the many other favorable agronomic traits of Shannong0096.

Recently, researchers have identified a few disease-resistant alien translocation lines with cytologically undetectable alien segments, which were called “cryptic alien introgressions” (Kuraparthi et al., 2007b; He et al., 2009; Luo et al., 2009). In the present study, two markers flanked \( YrSn0096 \) on the long arm of chromosome 4A, whereas GISH mapped a small alien segment to the short arm of a wheat chromosome. It suggested that Shannong0096 has two alien segments: one detected by GISH in the distal region of the short arm of a wheat chromosome that did not carry \( YrSn0096 \), and the other a cryptic introgression located on 4AL carrying the resistance gene. The presence of these alien segments is probably the reason for the seeming inconsistency between the GISH results and the microsatellite data.

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