Development of Single Nucleotide Polymorphism Markers for the Wheat Curl Mite Resistance Gene Cmc4

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ABSTRACT
Wheat curl mite (Aceria tosichella Keifer) is an important wheat (Triticum aestivum L. em. Thell.) pest in many wheat-growing regions worldwide. Mite feeding damage not only directly affects wheat yield, but A. tosichella also transmits Wheat streak mosaic virus (WSMV). Wheat resistance to A. tosichella, therefore, helps control WSMV. OK05312 (PI 670019) is an advanced breeding line released from Oklahoma that shows a high level of A. tosichella resistance. To map the gene(s) conditioning wheat resistance to A. tosichella in OK05312, a genetic linkage map was constructed using single nucleotide polymorphism (SNP) markers derived from genotyping-by-sequencing (GBS) and a population of 186 recombinant inbred lines (RILs) from the cross ‘Jerry’ (PI 632433)/OK05312. Seedlings of both parents and the RIL population were infested by A. tosichella Biotype 1 in greenhouse experiments. One major quantitative trait locus was identified on the short arm of chromosome 6D, which corresponds to the previously reported gene Cmc4 for A. tosichella resistance. This gene explained up to 71% of the phenotypic variation and was delimited in a 1.7-Mb (~3.3-cM) region by SNPs 370SNP7523 and 370SNP1639. We successfully converted 12 GBS-SNPs into Kompetitive allele specific polymerase chain reaction (KASP) markers. Two of them tightly linked to Cmc4 were validated to be highly diagnostic in a US winter wheat population and can be used for marker-assisted breeding for incorporation of Cmc4 into new wheat cultivars.

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**The wheat curl mite** (Aceria tosichella Keifer) is a microscopic (70 × 250 μm), soft-bodied, yellow-white, elongated arthropod of the order Acari and family Eriophyidae. *Aceria tosichella* was first described from tulip bulbs (Liliaceae) by Keifer in 1938 and later reported from onion (*Allium cepa* L.), garlic (*Allium sativum* L.), and several grass species (Poaceae), including common wheat (*Triticum aestivum* L. em. Thell.) (Slykhuis, 1955; Connin, 1956). A single female can produce more than three million eggs in 60 d under ideal conditions (Navia et al., 2013). *Aceria tosichella* may cause complete leaf trapping when infestation occurs.
in young plants and cause mild rolling of leaf edges when infestations occur in older plants during spring (Staples and Allington, 1956).

More importantly, *A. tosichella* transmits *Wheat streak mosaic virus* (WSMV) (Slykhuis 1955), *Wheat mosaic virus* (WMoV), formerly known as *High Plains virus* (HPV) (Seifers et al., 1997; Skare et al., 2006; Hadi et al., 2011), *Brome streak mosaic virus* (BrSMV) (Görtz and Maiss, 1995), and *Triticum mosaic virus* (TriMV) (Seifers et al., 2008, 2009). Virus symptoms include yellowing and rosette leaves and stunted plants, which usually can be observed on winter wheat as it undergoes stem elongation after winter dormancy. Outbreaks of WSMV are more severe if *A. tosichella* infestation occurs earlier during vegetative growth stages under warm temperatures (Wegulo et al., 2008). Therefore, *A. tosichella* is one of the most important wheat pests in the Great Plains of the United States and Canada, as well as in many other wheat-producing countries of Asia, Australia, Europe, and South America (Slykhuis, 1955; Keifer, 1969; Shevchenko et al., 1970; Martin et al., 1984; Harvey et al., 1990, 2002; Conner et al., 1991; Navia et al., 2013).

Given that no single effective measure exists for control of *A. tosichella*, genetic resistance in wheat has proven to be the most economical and environmentally safe strategy for reducing yield losses due to the combined effects of resistance to *A. tosichella* and associated viruses (Smith, 1999). To date, several genes conferring *A. tosichella* resistance have been reported. Thomas and Conner (1986) reported the first *A. tosichella* resistance gene (*Cmc1*) to be transferred from *Aegilops tauschii* (Coss.) Schmal. [syn. *Ae. squarrosa* L.; *Triticum tauschii* (Coss.) Schmal.] to wheat chromosome 6D (Thomas and Conner, 1986; Whelan and Thomas, 1989). *Cmc2* resides on a translocation from *Agropyron elongatum* (Host) P. Beauv. in the same wheat 6DL chromosome (Martin et al., 1976; Whelan and Hart, 1988). *Cmc3* is a gene on the 1AL.1RS translocation of rye (*Secale cereale* L.) in the wheat cultivar ‘TAM 107’ (PI 495594) and the breeding line KS96WGRC40 (PI 604225) (Malik et al., 2003b). KS96WGRC40 also carries an *Ae. tauschii*-derived *A. tosichella* resistance gene, *Cmc4*, on the short arm of chromosome 6D (Malik et al., 2003a). Although *Cmc1*, *Cmc2*, and *Cmc4* are all on the chromosome 6D, they are independent loci (Malik et al., 2003a). In addition, several other unnamed genes have been reported: one from *Haynaldia villosa* (L.) Schur in a T6A-L-6VS translocation line (Chen et al., 1996), one from wheat–*Thinopyrum intermedium* (Podp.) Barkworth & DR Dewey partial amphiploids (Chen et al., 1998, 2003), and one in a wheat–*Thinopyrum ponticum* 6Ae/6DL Robertsonian translocation line (Thomas et al., 1998). More recently, a new gene, *Cmc6-TAM112*, has been mapped on 6DS from a Texas cultivar ‘TAM 112’ (PI 643143) (Dhakal et al., 2018). However, its relationship with other genes on chromosome 6D is unknown.

In a previous study, Malik et al. (2003a) used a F$_{2:3}$ population derived from the cross KS96WGRC40 (*Cmc4*)/‘Wichita’ (Clt 11952, *A. tosichella* susceptible) to locate *Cmc4* on the distal end of 6DS at a $\sim$10.5-cM interval between markers $X_{dlm141}$ and $X_{ksug8}$. However, those two markers are still too far from *Cmc4* and are not useful for marker-assisted selection (MAS). Another marker *Xwms904* was reported to be closely linked to *Cmc4*, but its primer sequence has been patented and is not publicly available for breeding selection (Malik et al., 2003a). In addition, they have not been validated in diverse genetic backgrounds. In the present study, we confirmed that the *A. tosichella* resistance gene in OK05312 is *Cmc4*, fine mapped *Cmc4* using genotyping-by-sequencing (GBS)-based single nucleotide polymorphism (GBS–SNP) markers, and further converted a set of closely linked GBS–SNPs into high-throughput Kompetitive allele specific polymerase chain reaction (KASP) markers (Semagn et al., 2013) for efficient incorporation of *Cmc4* into new wheat cultivars in breeding programs.

**MATERIALS AND METHODS**

**Plant Materials**

A population of 186 F$_{5:4}$ recombinant inbred lines (RILs) was developed from the cross of ‘Jerry’/OK05312 using single-seed descent. Jerry, developed by North Dakota State University, is a hard winter hexaploid wheat derived from the cross ‘Roughrider’/ND7571/‘Arapahoe’ and is susceptible to *A. tosichella* (Peel et al., 2004). OK05312 is an advanced hexaploid hard winter wheat breeding line with the pedigree TX93V5919/KS96WGRC40/OK94P549/KS96WGRC34 (PI 604219) and was developed by Oklahoma State University in cooperation with the USDA-ARS (Cox et al., 1999; Carver et al., 2016). It has better agronomic traits than KS96WGRC40 and was thus released as an agronomically improved source of *Cmc4* for *A. tosichella* resistance. The wheat cultivar ‘Jagger’ (PI 593688) was used as an *A. tosichella*-susceptible check and TAM 107, which contains *Cmc3*, as an *A. tosichella* Type 1 resistant check (Harvey and Martin, 1992; Harvey et al., 1997, Sears et al., 1997, Dhakal et al., 2017). Validation with KASP markers was conducted using a natural US winter wheat population included elite breeding lines from regional performance nurseries and newly released cultivars. OK05312 was included in the population as the positive control.

**Wheat Curl Mite Maintenance and Infestation**

Greenhouse experiments were started on 10 Mar. 2014 (Exp. I) and 25 Apr. 2014 (Exp. II) at Kansas State University, Manhattan, KS. Biotype 1 of *A. tosichella* was used for infestation because it is predominant in Kansas and used in several previous studies (Malik et al., 2003a, 2003b). The Biotype 1 colony originated from Tripp County, South Dakota, and was collected and supplied courtesy of Dr. Ada Szczepaniec, South Dakota State University, maintained on the wheat curl mite-susceptible wheat cultivar Jagger, and periodically verified by polymerase chain reaction (PCR) using the ITS1 marker (Malik, 2001). Wheat curl mite eggs are periodically periodically verified by polymerase chain reaction (PCR) using the ITS1 marker (Malik, 2001).
transferred to healthy plants to provide viruliferous wheat curl mites. For mite infestation, plants of the RIL population were grown in 72-cell germination trays containing Pro-Mix ‘Bx’ potting mix (Premier ProMix). Five plants per genotype were grown in each experiment without replication. A total of five plants per parent and control were planted in the first experiment, and 10 plants per parent and control were planted in the second experiment. To phenotype plant reaction to A. tosichella, the leaf whorls of five test plants of each RIL and control were infested with 10 viruliferous A. tosichella adult mites at the two-leaf stage, and plants were covered by a mite-proof cage made of 36-μm mesh and left undisturbed for 21 d at 24/20°C day/night, and a photoperiod of 14/10 h light/dark for development of mite and virus symptoms. Plants were scored individually for resistance or susceptibility at 21 d after mite infestation based on the degree of virus symptoms. Plants were scored as resistant, and plants with curled or symptom expression in the susceptible control plants. Plants with resistance or susceptibility at 21 d after mite infestation based on the degree of virus symptoms. Plants were scored individually for resistance or susceptibility at 21 d after mite infestation based on the degree of virus symptoms. Plants were scored as resistant, and plants with curled or symptom expression in the susceptible control plants. Plants with

**DNA Extraction**

Leaf tissue from each genotype was sampled at the two-leaf stage into 1.1-mL-deep well plates with each well containing a 3.2-mm stainless steel bead. The plates with tissue were freeze dried for 48 h in a freeze dryer (Thermo Fisher) and shaken in a mixer mill (Retsch) at 25 cycles s⁻¹ for 5 min. Genomic DNA was extracted using a modified cetyltrimethyl ammonium bromide method (Bai et al., 1999).

**Genotyping-by-Sequencing Library Construction and SNP Identification**

A GBS library was constructed for 186 RILs and two parents in two 96-well plates following Poland et al. (2012). Parental samples had three replications each. In brief, DNA samples were digested with the Post-HF (high fidelity) andMspI restriction enzymes (New England BioLabs), and ligated to barcoded adapters and a Y common adaptor using T4 DNA ligase (New England BioLabs). All ligation products in the two 96-well plates were pooled and subjected to enzyme-linked immunosorbent assays (ELISAs) for WSMV, using a protocol described previously (Chuang et al., 2017).

**Analysis of SSR Markers**

Three simple sequence repeat (SSR) markers, Xgdm141 and Xwms904 closely linked to Cmvd4 (Malik et al., 2003a) and Xcsn9 on rye chromosome arm 1RS, were analyzed to verify presence of Cmvd4 and absence of Cmvd3 in the RIL population. The PCR amplifications were performed in a Tetrad Peltier DNA engine (Bio-Rad Laboratories) following Malik et al. (2003a). The PCR products were separated on an ABI PRISM 3730 DNA analyzer (Applied Biosystems). The data were scored using GeneMarker (SoftGenetics, 2014).

**Linkage Map Construction and QTL Mapping**

A linkage map was initially constructed using SNPs generated from GBS using the ‘Regression’ function in JoinMap version 4.0 (Van Ooijen, 2006). Recombination fractions were converted to centimorgans using the Kosambi function (Kosambi, 1944). The linkage groups were assigned to chromosomes based on the previously published Chinese Spring reference genome RefSeq v1.0 by The International Wheat Genome Sequencing Consortium (IWGSC, 2018). Phenotypic data from the two experiments were separately analyzed for quantitative trait locus (QTL) detection, and mean infestation rates from the two experiments were also calculated for QTL detection. Quantitative trait locus mapping was conducted using composite interval mapping modules in QTL Cartographer version 2.5 (Wang et al., 2012). Significant LOD threshold of three was selected for all datasets based on 1000 permutations (Doerge and Churchill, 1996) with a Type I error rate of <0.05.

**Conversion of GBS-SNPs into KASP Markers**

Initially, a map of GBS-SNPs was used to identify QTL location. To fill in missing GBS data in detected QTL region, GBS-SNPs mapped around the QTL region were selected for conversion of KASP markers. The KASP primers were designed using the Polymarker pipeline (http://polymarker, tgac.ac.uk/) that designs homoeologue-specific KASP assays for the polyploid wheat genome. The KASP assay was performed following manufacturer’s instruction (http://www.lgcgroup.com/LGCGroup/media/PDFs/Products/Genotyping/KASP-genotyping-chemistry-User-guide.pdf). The newly designed KASP primers were then tested for parental polymorphisms, and the polymorphic SNPs were genotyped in the mapping population. The new linkage map was reconstructed for final QTL analysis after the newly developed polymorphic KASP-SNPs replaced their corresponding GBS-SNPs.

The KASP assay was performed in a 6-μL PCR mix that consisted of 2.9 μL of reaction mix (LGC Genomics), 0.1 μL of
primer assay mix, and 3 μL of DNA at a concentration of 15 ng μL−1. Polymerase chain reaction was assayed following manufacturer’s instruction (LGC Genomics) using an ABI 7900HT real-time PCR system (Life Technology).

RESULTS
Wheat Curl Mite Resistance in the Jerry/OK05312 RIL Population
All parents and controls were infested with A. tosichella US Biotype 1. However, the percentage of infested plants differed between the susceptible genotypes (the susceptible control Jagger and the susceptible parent Jerry) and the resistant genotypes (the resistant parent OK05312 and the resistant control TAM 107). Jagger showed the highest mean infestation rate with 87% susceptible plants, Jerry the second with 67% susceptible plants, and OK05312 and TAM 107 the lowest with only 7% infested plants over the two experiments. The difference between the resistant and susceptible parents was significant (p < 0.01). The frequency distribution of percentage of infested plants in the RIL population was continuous with ?50% of the RILs being the resistant genotypes that had at least 90% resistant plants per RIL (Fig. 1), suggesting that one major gene may confer A. tosichella resistance in OK05312. In addition, transgressive segregation was also observed for resistance.

Construction of a Linkage Map with GBS-SNPs for QTL Mapping
Genotyping-by-sequencing generated a total of 13,730 SNPs. Among them, 2048 SNPs had <20% missing data, and 1526 of them were mapped into 46 linkage groups that were anchored to 21 chromosomes. The linkage map has a total length of 2369 cM with an average marker density of 1.55 cM per marker and 5 to 130 markers per linkage group.

One major QTL associated with A. tosichella resistance and low value of ELISA was identified in each experiment and for mean from the two experiments. The QTL was located on the short arm of chromosome 6D, with OK05312 providing the allele for A. tosichella resistance (Fig. 2).

Conversion of GBS-SNPs to Enabling KASP Markers
To verify the SNP data generated by GBS and fill in missing data for the markers in the QTL region, 20 sequences carrying GBS-SNPs mapped in the distal end of 6DS, where the A. tosichella resistance QTL was located, were used to design KASP assays to screen two parents for polymorphisms. Twelve KASP-SNPs were polymorphic between the parents (Table 1) and showed similar segregation patterns to their corresponding GBS-SNPs in the RIL population with zero to three mismatches between KASP and GBS data per marker in 186 lines analyzed. Among those KASP-SNP markers, four markers had a mismatch in one RIL; four had two mismatches in two different RILs; one (370SNP2013) had three mismatches in three different RILs; and three had perfect matches. The 12 KASP-SNPs were remapped to (or close to) the original positions in the map because of a low average mismatch rate (0.68%) between GBS-SNPs and KASP-SNPs. The 15 mismatched SNP calls between KASP and

Fig. 1. Frequency distribution for the percentage of wheat curl mite resistant lines in the recombinant inbred line (RIL) population of Jerry/OK05312 evaluated in two experiments. Mean refers to average from the two experiments (Exp. I and Exp. II).
Fig. 2. A partial linkage map for wheat curl mite resistance quantitative trait loci (QTLs) identified on the distal end of chromosome arm 6DS. Genetic distances in centimorgans are shown on the left side linkage map and marker names are shown on the right. The QTL data from Exp. I, Exp. II, mean, and enzyme-linked immunosorbent assay (ELISA) are plotted by red, black, blue, and green lines, respectively. Bars for the QTL positions from left to right are calculated using data from Exp. I, Exp. II, mean, and ELISA, respectively. The numbers on the top of the QTL plot are logarithm of the odds (LOD) values.

Table 1. List of primers for polymorphic Kompetitive allele specific polymerase chain reaction (KASP)-single nucleotide polymorphism (SNP) markers developed in this study and their estimated physical distances in the Chinese Spring wheat reference genome (IWGSC, 2018) and the A. tauschii reference genome (Luo et al., 2018).

<table>
<thead>
<tr>
<th>SNP markers</th>
<th>Forward primers (Jerry/OK05312)†</th>
<th>Reverse primers</th>
<th>Physical distance in Chinese Spring bp</th>
<th>Physical distance in A. tauschii bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>370SNP0898</td>
<td>TCTGCTACTCAAATGCATG/g</td>
<td>CGGCTGTATGTTGTAAC</td>
<td>1,602,580–1,602,643</td>
<td>1,418,676–1,418,739</td>
</tr>
<tr>
<td>370SNP1205</td>
<td>CCCTTCTGACGCTTGATc</td>
<td>AGCTTTGCTCCCCAACGCTC</td>
<td>2,325,387–2,325,449</td>
<td>2,000,396–2,000,523</td>
</tr>
<tr>
<td>370SNP6314</td>
<td>CGCCCTTCTGACGCTTGATc</td>
<td>AGCTTTGCTCCCCAACGCTC</td>
<td>2,325,379–2,325,436</td>
<td>2,000,466–2,000,523</td>
</tr>
<tr>
<td>370SNP2013</td>
<td>GTTATATGTCGATGTTGTC/g</td>
<td>ACGGGCGTCACTAACAC</td>
<td>2,523,758–2,523,821</td>
<td>No match sequence</td>
</tr>
<tr>
<td>370SNP7523</td>
<td>TTCCGATCCAGCCACAc</td>
<td>GCTGCTGAGAATTTTGTTS</td>
<td>2,240,736–2,240,799</td>
<td>2,101,778–2,101,841</td>
</tr>
<tr>
<td>370SNP1060</td>
<td>CAGGCAGGATGCATTTT/c</td>
<td>CTGGGAGCTGGTTTAG</td>
<td>2,914,472–2,914,535</td>
<td>2,818,042–2,818,105</td>
</tr>
<tr>
<td>370SNP102</td>
<td>GACGACACACAGAGAGAAGa</td>
<td>GCTGCCGCGTCTTTGTT</td>
<td>3,041,197–3,041,260</td>
<td>2,874,075–2,874,138</td>
</tr>
<tr>
<td>370SNP1639</td>
<td>GTGCAGTGTACGCGGACg/a</td>
<td>GTTTCGGCAACTCTACGC</td>
<td>3,912,903–3,912,966</td>
<td>No match sequence</td>
</tr>
<tr>
<td>370SNP755</td>
<td>TGCCAGCCACACAGAGACAc</td>
<td>CGCATGAAAGACAGTATG</td>
<td>5,046,882–5,046,945</td>
<td>4,801,965–4,802,027</td>
</tr>
<tr>
<td>370SNP1714</td>
<td>CTGCCATGCTCGATCA/c</td>
<td>GAGTGCAATAGACTGACTGATGAGGTGTTG</td>
<td>5,255,879–5,255,942</td>
<td>4,818,680–4,818,744</td>
</tr>
<tr>
<td>370SNP1705</td>
<td>CTTGATGCGAGGAGCCa/c</td>
<td>GCTGATGCGCACAACACT</td>
<td>6,106,322–6,106,385</td>
<td>5,361,409–5,361,472</td>
</tr>
<tr>
<td>370SNP776</td>
<td>GGCATGTGAAGGTCAGCTTg/a</td>
<td>GCCATCTGCTCATCAAGTAA</td>
<td>6,343,670–6,343,728</td>
<td>5,687,936–5,687,994</td>
</tr>
<tr>
<td>370SNP1094</td>
<td>GTGCAATCAAGGCCAGGg/c</td>
<td>CAGCTCTATCGCAACCACA</td>
<td>7,172,887–7,172,950</td>
<td>6,446,854–6,446,917</td>
</tr>
</tbody>
</table>

† Last two letters separated by a backslash indicate the polymorphic nucleotides in Jerry (the letter before the slash) and OK05312 (after the back slash).
GBS were present in six RILs, including five in RIL179, four in RIL141, three in RIL147 and one each in RILs 78, 155, and 178.

After the map was updated with the KASP markers, the major QTL for *A. tosichella* resistance was delimited to a 3.3-cM interval between KASP markers 370SNP7523 and 370SNP1639 and explained 71.31% and 30.58% of the phenotypic variation in the two experiments, respectively, and 49.94% of the phenotypic variation for the mean of the two experiments (Table 2, Fig. 2). A QTL from ELISA data was also located in this region and explained 35.72% of the phenotypic variation. Therefore, the 3.3-cM chromosome region between markers 370SNP7523 and 370SNP1639 is the critical region for curl mite resistance in OK05312.

To validate the usefulness of the two KASP markers (370SNP7523 and 370SNP1639) in MAS, they were analyzed for allele distribution in a wheat population. This population include both hard and soft US winter wheat cultivars and breeding lines. Both markers amplified the positive alleles in accessions NW03666 and OK05312, not in the other accessions, except that 370SNP1639 has the positive allele in HV9W96-1271R-1 (Supplemental Table S1). Those results suggest a high level of polymorphism of the two markers between OK05312 and other US winter wheat cultivars and elite breeding lines.

### Annotated Putative Genes in the Cmc4 Flanking Region

A Basic Local Alignment Search Tool (BLAST) search using the sequences of the two flanking markers for the mite resistance QTL located a physical distance of 1672 kb between the flanking markers 370SNP7523 (2241 kb) and 370SNP1639 (3913 kb, Table 1). A total of 55 putative genes were predicted in this region using Chinese Spring RefSeq v1.0 (IWGSC, 2018). Six were annotated as disease resistance genes, including a nucleotide binding site (NBS)-leucine-rich repeat (LRR)-like resistance protein, three LRR receptor-like protein kinase family proteins, a protein-enhanced disease resistance 2-like protein, and a Toll interleukin-1 receptor (TIR)-NBS-LRR class disease resistance protein. Using the *Aegilops tauschii* reference (Luo et al., 2018), at least 34 genes were annotated with a high confidence in the syntenic region (Supplemental Table S2), and two of them are putative disease resistance genes (a protein-enhanced disease resistance 2-like protein and a TIR-NBS-LRR class disease resistance protein). Those two putative resistance genes were also found in the syntenic region of the hexaploid wheat Chinese Spring reference (IWGSC, 2018).

### Analysis of DNA Markers Linked to Cmc4 in the RIL Population

Blast search of these SNP sequences presented in Table 2 against the Chinese Spring wheat reference sequence indicated that the newly identified QTL is on the distal end of short arm of chromosome 6D. To verify if the QTL was Cmc4, two previously reported DNA markers were genotyped in the RIL population: Xwms904 was the closest marker to Cmc4, and Xgdm141 was one of the flanking markers (Malik et al., 2003a). Xwms904 amplified a target fragment of 115 bp in Jerry and did not amplify any PCR product in OK05312. Xgdm141 amplified a target band of 147 bp in OK05312 and 127 bp in the Jerry. When these markers were analyzed together with GBS–SNP data, Xwms904 was 1.3 cM from 370SNP1639, one of the flanking markers for the QTL mapped in Jerry × OK05312 population, confirming that the QTL in OK05312 is Cmc4. Xgdm141 was mapped at 42.4 cM to 370SNP1639; therefore, the newly developed flanking markers for Cmc4, 370SNP7523 and 370SNP1639, are closer to Cmc4 than Xwms904 and Xgdm141.

### DISCUSSION

To date, only four named (*Cmc1*, *Cmc2*, *Cmc3*, and *Cmc4*) and two unnamed *A. tosichella* resistance genes have been reported and transferred into wheat from wheat relative species (Thomas and Whelan, 1991; Chen et al., 1996, 1998, 2003; Malik et al., 2003a; Dhakal et al., 2018), and KS96WGRC40 carries two of them, *Cmc4* and *Cmc3*.
Four KASP-SNPs were identified within the Cmc4 interval. Identification of these closely linked SNPs to Cmc4 provides useful markers for further fine mapping or cloning the gene. Previous deletion mapping of the linked markers to the gene showed that the Ae. tauschii-derived fragment carrying Cmc4 is in the distal end of chromosome arm 6DS within the bin interval 0.99 to 1.00 (Malik et al., 2003a). Physical mapping of Cmc4 using Chinese Spring reference delimited the gene to a 1.7-Mb interval between 2.2 and 3.9 Mb, suggesting that the fragment carrying Cmc4 is short, is on the distal end of 6DS, and can therefore be easily transferred from OK05312 into new wheat cultivars. OK05312 is a commercial-ready genetic stock, shows resistance to both curl mite biotypes in the US Great Plains, and could be directly released as a variety. However, commercially wide-scale deployment of Cmc4 as a unilateral defense to WSMV may not be a good example of responsible gene stewardship (Carver et al., 2016); thus, pyramiding Cmc4 with other resistance genes in new cultivars may provide durable resistance to WSMV.

Previously available markers linked to Cmc4 were mainly SSR and restriction fragment length polymorphism (RFLP) markers (Malik et al., 2003a). An RFLP marker is not suitable for practical breeding application due to its low throughput and technical complexity. For two previously reported SSR markers, Xgdm141, one of the flanking marker of Cmc4, is too far from Cmc4 (>42 cM) in the current study, whereas other SSR marker, Xwms904 (Malik et al., 2003a), has been patented and is not publicly available. To effectively use Cmc4 in breeding, tightly linked and high-throughput markers are needed. In this study, many GBS-SNPs were mapped in the gene region, which significantly shortened the region and more precisely pinpointed the gene interval to 1.7 Mb. However, GBS-SNPs are still not suitable for screening a large number of breeding samples due to high technical demand and relatively high cost per sample. The KASP marker has the advantages of easy assaying and low cost per sample and is also suitable for high-throughput screening. To develop enabling markers for breeding applications, we successfully converted 12 SNPs that segregated in the RIL population. Among them, 370SNP7523 and 370SNP1639 flank Cmc4 in a natural population of US winter wheat cultivars and breeding lines. In this population, Cmc4 is not expected according to their known pedigrees (Supplemental Table S1). The two markers identified only one line as positive except the OK05312 control; therefore, those markers are nearly diagnostic and should be very useful for MAS to select for Cmc4 in breeding programs.

Candidate gene analysis using both Chinese Spring and A. tauschii reference sequences identified 55 and 34 annotated high confidence genes in the putative Cmc4 region. Among them, two putative resistance genes, a protein-enhanced
disease resistance 2-like protein, and a TIR-NBS-LRR class disease resistance protein were identified in both genomes. They can be considered as important candidates for further map-based cloning of Cmc4.

Supplemental Material Available
Supplemental material is available online for this article.

Conflict of Interest
On behalf of all authors, the corresponding author states that there is no conflict of interest.

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