

Introgression of a Novel Ug99-Effective Stem Rust Resistance Gene into Wheat and Development of *Dasypyrum villosum* Chromosome-Specific Markers via Genotyping-by-Sequencing (GBS)

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Abstract

Dasypyrum villosum is a wild relative of common wheat (*Triticum aestivum* L.) with resistance to *Puccinia graminis* f. *tritici*, the causal agent of stem rust, including the highly virulent race TTKSK (Ug99). In order to transfer resistance, *T. durum*–*D. villosum* amphiploids were initially developed and used as a bridge to create wheat–*D. villosum* introgression lines. Conserved ortholog set (COS) markers were used to identify *D. villosum* chromosome introgression lines, which were then subjected to seedling *P. graminis* f. *tritici* resistance screening with race TTKSK. A COS marker-verified line carrying chromosome 2V with TTKSK resistance was further characterized by combined genomic in situ and fluorescent in situ analyses to confirm a monosomic substitution

line MS2V(2D) (20" + 1' 2V[2D]). This is the first report of stem rust resistance on 2V, which was temporarily designated as *SrTA10276-2V*. To facilitate the use of this gene in wheat improvement, a complete set of previously developed wheat–*D. villosum* disomic addition lines was subjected to genotyping-by-sequencing analysis to develop *D. villosum* chromosome-specific markers. On average, 350 markers per chromosome were identified. These markers can be used to develop diagnostic markers for *D. villosum*-derived genes of interest in wheat improvement.

Keywords: cereals and grains, field crops, fungi, techniques.

Dasypyrum villosum (L.) P. Candargy (2n = 2x = 14, VV) is an outcrossing tertiary gene pool relative of wheat (*Triticum aestivum* L.). The species originated around the northeastern Mediterranean and Caucasus region (De Pace et al. 2011) and has been utilized for wheat improvement due to abiotic stress tolerance, including salt and drought, as well as resistance to several wheat diseases, including

powdery mildew, eyespot, loose smuts, take-all disease, leaf spot, wheat curl mite, *Wheat spindle streak mosaic virus*, leaf rust, and stem rust (De Pace et al. 2011; Grądzielewska 2006). A collection of *D. villosum* accessions (*n* = 95) was previously screened, which revealed near-immunity of this species to wheat stem rust, caused by *Puccinia graminis* f. *tritici* Erikss. & Hennings (Pumphrey et al. 2008).

Stem rust has reemerged as a threat to world wheat production since the emergence of a new virulent race, TTKSK (also known as Ug99), and evolution of related races (Singh et al. 2011, 2015). Stem rust has been effectively controlled by using resistance genes throughout the world in recent decades; however, the Ug99 race group defeated many effective wheat stem rust resistance (*Sr*) genes and continues to evolve (Jin et al. 2008, 2009; Newcomb et al. 2016). The Ug99 race group is virulent to the majority of genes of common wheat origin. There are at least 41 *Sr* genes effective against the Ug99 race group to date (Hiebert et al. 2016; Rahmatov et al. 2016; Singh et al. 2015). Thirteen genes are derived from common wheat backgrounds and the remaining are derived from other species belonging to the primary, secondary, and tertiary gene pools. One of the resistance genes, *Sr52*, was identified on *D. villosum* chromosome 6VL via a Robertsonian translocation (T6AS-6V#3L) conferring resistance to TTKSK (Qi et al. 2011). *Sr52* is the only *Sr* gene derived from *D. villosum*. It is important to broaden the genetic basis of stem rust resistance genes by acquiring new resistance genes from other gene pools, not only to control the disease but also to prolong the durability of the genes by pyramiding (McDonald and Linde 2002).

Alien gene transfer for disease resistance control in wheat is not a new concept. In order to transfer resistance genes from *D. villosum* to wheat, addition, substitution, and translocation lines were developed (Grądzielewska 2006). Creating these lines required specialized techniques, including embryo rescue and chromosome

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doubling techniques. To minimize the size of the transferred chromosome segments to reduce the potential for linkage to undesirable alleles on the same segments, inducing homologous recombination of chromosomes harboring resistance genes and developing chromosome-specific single-nucleotide polymorphism (SNP) markers spanning target chromosomes will help track alien resistance in a wheat background. Also, it provides markers for targeted chromosome engineering.

D. villosum is highly heterogeneous due to open pollination; therefore, it is challenging to develop adequate markers (De Pace et al. 2011; Qi et al. 2011). However, with the advances in molecular techniques, additional chromosome-specific markers were developed for genotyping (Cao et al. 2009; He et al. 2013; Liu et al. 2011; Qi et al. 2011; Zhang et al. 2006; R. Zhang et al. 2017; X. Zhang et al. 2017). Conserved ortholog set (COS) markers are developed based on genes which are highly conserved and low copy across a species and are suitable for comparisons to closely related species (Molnár et al. 2013; Pont et al. 2013; Quraishi et al. 2009). In total, 695 COS markers were developed based on rice-wheat orthologs and these markers are available for cereal genome research (Quraishi et al. 2009).

Genotyping-by-sequencing (GBS) is a recent high-throughput method of genotyping species with no reference genomes (Poland and Rife 2012; Poland et al. 2012). GBS reduces the genome information by using restriction enzymes targeting intron and exon regions to capture the polymorphic regions. This approach is especially suitable for species such as *D. villosum* with limited sequence information, because it can provide de novo sequence and marker information simultaneously.

In this study, we created *T. durum*–*D. villosum* amphiploids from four *D. villosum* accessions. These amphiploids were screened with TTKSK and the resistant lines were advanced to develop wheat–*D. villosum* single-chromosome introgression lines. A wheat–*D. villosum* monosomic substitution line containing *D. villosum* chromosome 2V conferred TTKSK resistance. To facilitate the transfer of this new stem rust resistance gene to wheat, GBS was used to genotype a complete set of Chinese Spring (CS)–*D. villosum* addition lines to anchor *D. villosum* sequence tags on each chromosome.

Materials and Methods

Plant material. *D. villosum* accessions and a complete chromosome series of wheat–*D. villosum* disomic additions DA1V#3

(TA7677) through DA7V#3 (TA7683) and a set of Robertsonian translocations (cRobTs) involving 12 of the 14 *D. villosum* chromosomes were obtained from the Kansas State University Wheat Genetic Resource Center (Table 1). Tetraploid *T. durum* ‘Rusty’ (PI 639869) (Klindworth et al. 2006), susceptible to all *P. graminis* f. *tritici* races tested in North America and races of exotic origin, was used as the female in crosses with *D. villosum* to make amphiploids. A population of F_{2,3} families of amphiploids Rusty/TA10276B//Rusty//TA2126B (Table 2) were created for allelism testing. CS was used for subsequent crosses with amphiploids to produce wheat–*D. villosum* single-chromosome introgression lines. Initially, the amphiploids were used as female plants and CS was used as the pollen donor to produce F₁ seed. These F₁ plants were backcrossed to CS as female to create BC₁F₁ progeny. The BC₁F₁ progeny were further backcrossed to CS or self-pollinated until representative single *D. villosum* introgression lines were isolated. *D. villosum* accession TA10260 was used for DNA extraction to produce a *D. villosum*-specific genomic in situ hybridization (GISH) probe; and TA5617, a 6AS·6VL translocation line, was used as a GISH-positive control.

Embryo rescue and colchicine treatment. Immature embryos of Rusty/*D. villosum* hybridizations were removed from the spikes 14 days after pollination. Immature embryos were sterilized with 20% commercial bleach solution (containing 6% NaOCl) for 20 min followed by rinsing with sterilized water three times in a laminar hood. Immature embryos were isolated under a dissecting scope. The embryos were placed on wheat embryo rescue (ER) medium containing Murashige and Skoog (MS) basal salt mixture

Table 2. Parental and Rusty-*Dasyphyrum villosum* amphiploid infection types in response to *Puccinia graminis* race TTKSK at seedling stage

<i>D. villosum</i> accessions	Origin	Infection type ^a		
		Parent	A	B
TA2126	Croatia	0;	13-	0;
TA2197	Bosnia	0;	0;	0;
TA10260	Greece	0;	0;1	-
TA10276	Greece	0;	3+	2

^a A and B represent different primary amphiploid plants. Seedling infection types were scored based on a 0-to-4 scale by (Stackman et al. 1962).

Table 1. List of genotypes used for this study

Genotype	Genetic stocks	Description ^a	Reference
TA2197	<i>Dasyphyrum villosum</i>	<i>D. villosum</i>	...
TA2126	<i>D. villosum</i>	<i>D. villosum</i>	...
TA10276	<i>D. villosum</i>	<i>D. villosum</i>	...
TA10260	<i>D. villosum</i>	<i>D. villosum</i>	...
TA7677	DA1V	CS- <i>D. villosum</i> disomic addition 1V#3	Lukaszewski, unpublished
TA7678	DA2V	CS- <i>D. villosum</i> disomic addition 2V#3	...
TA7679	DA3V	CS- <i>D. villosum</i> disomic addition 3V#3	...
TA7680	DA4V	CS- <i>D. villosum</i> disomic addition 4V#3	...
TA7681	DA5V	CS- <i>D. villosum</i> disomic addition 5V#3	...
TA7682	DA6V	CS- <i>D. villosum</i> disomic addition 6V#3	...
TA7683	DA7V	CS- <i>D. villosum</i> disomic addition 7V#3	...
TA5616	T1DL·1V#3S	cRobT CS- <i>D. villosum</i> 1DL/1VS	Liu et al. (2011)
TA5615	T1DS·1V#3L	cRobT CS- <i>D. villosum</i> 1DS/1VL	...
TA5634	T2BS·2V#3L	cRobT CS- <i>D. villosum</i> 2BS/2VL	...
TA5636	T3DL·3V#3S	cRobT CS- <i>D. villosum</i> 3DL/3VS	...
TA5637	T3DS·3V#3L	cRobT CS- <i>D. villosum</i> 3DS/3VL	...
TA5595	T4DL·4V#3S	cRobT CS- <i>D. villosum</i> 4DL/4VS	...
TA5594	T4DS·4V#3L	cRobT CS- <i>D. villosum</i> 4DS/4VS	...
TA5638	T5DL·5V#3S	cRobT CS- <i>D. villosum</i> 5DL/5VS	...
TA5618	T6AL·6V#3S	cRobT CS- <i>D. villosum</i> 6AL/6VS	...
TA5617	T6AS·6V#3L	cRobT CS- <i>D. villosum</i> 6AS/6VL	...
TA5639	T7DL·7V#3S	cRobT CS- <i>D. villosum</i> 7DL/7VS	...
TA5640	T7DS·7V#3L	cRobT CS- <i>D. villosum</i> 7DS/7VL	...
Rusty (R)	PI 639869	<i>Triticum turgidum</i>	Klindworth et al. (2006)
CS	TA3803	<i>T. aestivum</i>	...

^a CS = Chinese Spring and cRobT = Robertsonian translocation.

(PhytoTechnology Laboratory, Shawnee Mission, KS, U.S.A.), 1% Gamborg B5 vitamins (PhytoTechnology Laboratory), 3% sucrose (bioWORLD, Dublin, OH, U.S.A.), kinetin at 2 mg·liter⁻¹ (Sigma-Aldrich, St. Louis, MO, U.S.A.), 0.2% 2-(N-morpholino)ethanesulfonic acid (MES; PhytoTechnology Laboratory), and 0.2% Gelrite (Plant-Media, Dublin, OH, U.S.A.) with pH 5.7 adjusted by KOH (Sigma-Aldrich) in a growth room with a 16-h photoperiod at 24°C. After embryos were germinated and formed several roots on the ER medium, seedlings were subcultured to shoot elongating medium which contains MS basal salt mixture, 1% Gamborg B5 vitamins, 4% maltose (PhytoTechnology Laboratory), 0.01% ascorbic acid (Sigma-Aldrich), 0.2% MES, and 0.2% Gelrite with pH 5.7 until several tillers were formed. Seedlings were removed from the medium and transplanted into pots with potting medium that was covered with clear plastic and allowed to gradually reduce humidity. Once four to five leaves were formed, roots were cut about 2.5 cm and rinsed with distilled water to remove the potting medium. Subsequently, these seedlings were treated with 0.1% colchicine (Sigma-Aldrich) with 2% dimethyl sulfoxide (Sigma-Aldrich) for 7 h with light, then rinsed with running water for 20 h. Rinsed plants were placed in the beaker and roots of the rinsed plants were submerged in distilled water, refrigerated for 3 days with 16 h of light, and covered with plastic wrap to avoid desiccation. After plants recovered from the colchicine treatment, they were transplanted in the potting medium and grown in the greenhouse.

Stem rust screening. TTKSK screening was conducted at the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) Cereal Disease Laboratory in St. Paul, MN. Seedlings (7 to 9 days old) were inoculated with *P. graminis* f. *tritici* race TTKSK (isolate 04KEN156/04) using the protocol described by Rouse et al. (2011). Briefly, 7-day-old seedlings were inoculated with *P. graminis* f. *tritici* urediniospores suspended in Soltrol 70 oil. After inoculation, plants were air dried to evaporate the oil for at least 30 min. Subsequently, inoculated plants were placed in a dew chamber at 22°C in darkness with 100% humidity for 16 h. After 16 h, fluorescent and incandescent lights were illuminated inside the dew chamber for 4 h to promote *P. graminis* f. *tritici* penetration. Plants were removed from the dew chamber and slowly dried, then placed in a growth chamber at 22 and 18°C (day and night, respectively) with a 16-h photoperiod. At least 18 to 20 plants were tested from each line. Twenty North American resistance gene differential genetic stocks were included to confirm the *P. graminis* f. *tritici* race in each experiment. Fourteen days post inoculation, plants were scored for seedling infection types using the 0-to-4 scale described by Stakman et al. (1962).

DNA extraction and genotyping. Genomic DNA was extracted from freeze-dried 10-day-old plants using a BioSprint 96 DNA plant kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's protocol. DNA quality and quantity were assessed by Nanodrop. DNA concentration was adjusted to 25 ng/μl. COS markers developed by Quraishi et al. (2009) and *D. villosum* chromosome arm-specific markers (He et al. 2013; X. Zhang et al. 2017) were modified with the addition of the M13 tail (5'-CACGACATTGTAAAC GAC) on each forward primer. In each 12-μl reaction, 25 ng of template DNA, 1.2 μl of 10× NEB polymerase chain reaction (PCR)

buffer with 15 mM of MgCl₂ (New England BioLabs Inc. [NEB], Ipswich, MA, U.S.A.), 1.2 μl of 2 mM dNTP (Promega Corp., Madison, WI, U.S.A.), 0.48 μl of 25 mM MgCl₂ (NEB), 0.3 μl of 10 μM reverse primer, 0.06 μl of 0.1 μM reverse primer, 0.24 μl of 10 μM M13 primer labeled with one of four fluorescent dyes (FAM, VIC, NED, and PED), and 0.2 μl of Taq (NEB) were used. A touchdown PCR method was used as follows. Initial denaturing was at 94°C for 5 min; 12 touchdown cycles were performed at 95°C for 45 s, 64°C for 30 s, and 72°C for 1 min with 0.5°C decrease per cycle; followed by 35 amplification cycles at 95°C for 45 s, 58°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. An ABI 3730x Genotyper (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was used to process the PCR products. Allele calling was processed by GeneMarker V1.91.

GBS was performed at the USDA-ARS Western Regional Small Grains Genotyping Lab, Pullman WA, following the method described by Poland et al. (2012) and Truong et al. (2012). Genomic DNA was extracted as described previously. Quality and quantity of DNA was analyzed by a Synergy 2 microplate reader (Biotek, Winooski, VT, U.S.A.) using a fluorescent DNA quantitation kit (Bio-Rad, Hercules, CA, U.S.A.) and adjusted to 20 ng/μl. Two restriction enzymes (*Pst*I and *Msp*I; NEB) were used and a unique barcode adaptor was ligated for multiplexing. After the libraries were pooled, a DNA fragment was selected with 2% size select e-gel and examined for size and molarity with Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.) with the high sensitivity DNA kit. Chip P1v2 and Ion Proton IC 200 kits (Thermo Fisher Scientific) were used to process the libraries for Ion Proton System sequencing.

Selected *D. villosum* chromosome-specific GBS markers were verified by PCR. Primers were designed using Primer-BLAST (Ye et al. 2012). Parameters used for primer design were set to primer size between 15 to 25 bp, primer melting temperature between 57 and 63°C, and maximum product size of 90 bp. In each 20-μl reaction, 25 ng of template DNA, 2 μl of 10× PCR buffer (Qiagen), 4 μl of 5× Q-solution (Qiagen), 2 μl of 2 mM dNTP (Promega Corp.), 0.5 μl of 10 μM primers, and 0.2 μl of HotStart Taq DNA polymerase (Qiagen) were used. PCR conditions were as follows: after initial denaturing at 95°C for 15 min, 10 touchdown cycles at 95°C for 30 s, 65°C for 30 s, and 72°C for 10 s with 0.5°C decrease per cycle;

Table 4. Infection type (IT) of *Dasypyrum villosum* single-chromosome introgression lines in response to *Puccinia graminis* race TTKSK at seedling stage

Chromosome	Generation	Number of plants ^a	
		IT;2-	IT3+
1V	BC ₃ F ₂	...	20
2V	BC ₂ F ₂	10 ^b	10
3V	BC ₄ F ₁	...	19
4V	BC ₃ F ₂	...	20
5V	BC ₃ F ₂	...	18
6V	BC ₁ F ₂	...	20

^a Infection types at the seedling stage based on a 0-to-4 scale by Stakman et al. (1962).

^b Number of plants tested had respective TTKSK phenotype.

Table 3. Primer sequences of *Dasypyrum villosum* chromosome specific conserved ortholog set (COS) markers

Marker ^a	Chr. ^b	Forward primer ^c	Reverse primer
GPI:C:749284	1V	CTACGTGATTTTCTGGGTGG	TGCACACCTTCACTCTCTGG
GPI:C:753773	2V	GGACAATGAGAAAGCAAAGG	CTTTGCAAGAGCATCAGAGG
GPI:C:757080	3V	AGTGCAAGTCTCTGTGGAGG	TCATTGAAACCAAGGACAGC
GPI:C:765452	4V	CCTCTTTCAGAAAGGAAAGC	GTACACCGCGTAGATGATCC
GPI:C:717465	5V	TATATTGCTTGGACATCTTTGG	GTTCACTAGCATGCCTTTCC
GPI:C:749072	6V	GTCTTCACATTGATCCCAGC	TCTCAAGAACCTACCTGCC
GPI:C:744741	7V	CATTGACTCTAGACGGTGTGC	TATGGTTCTTCAGCTTTGC

^a Markers were developed by Quraishi et al. (2009).

^b *D. villosum* chromosome.

^c M13 tail was added to forward primer.

followed by 30 amplification cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 10 s; and a final extension at 72°C for 5 min. The PCR products were separated in 2% agarose gels in Tris-borate-EDTA buffer and visualized by ethidium bromide staining under UV light.

Cytogenetic analysis. Initially, the presence of *D. villosum* chromatin in the TTKSK-resistant introgression lines was verified by GISH. Ten plants from each line were analyzed. The squash preparations and GISH procedure were performed according to Zhang et al. (2001), except that no *T. aestivum* blocking DNA was applied. To determine the homologous group of *D. villosum* chromosomes, multicolor fluorescence in situ hybridization (FISH) combined with GISH was used. Individual wheat chromosomes were identified by FISH staining of tandem repeats and *D. villosum* chromosomes were detected by GISH. Somatic chromosome preparations using the drop technique, direct probe labeling by nick translation, and the FISH procedure were performed according to Kato et al. (2006), with minor modifications, as previously described (Danilova et al. 2012). Genomic DNA was labeled with fluorescein-12-dUTP (catalog NEL413001EA; Perkin-Elmer, Waltham, MA); oligonucleotide probes Cy5-(GAA)_n and TEX615-pAs1-2 (Danilova et al. 2012) were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (catalog number H-1200; Vector Laboratories, Burlingame, CA). Images were captured with a Zeiss Axioplan 2 microscope using camera CoolSNAP HQ2 (Photometrics,

Tucson, AZ U.S.A.) and AxioVision 4.8 software (Carl Zeiss AG, Oberkochen, Germany) and processed using the Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, U.S.A.).

Bioinformatics analysis. The GBS tags that corresponded to Rusty and the CS reference sequence were assembled by combining five replicate sequencing runs from DNA extracted from distinct plants of each genotype. Similarly, sequences from four accessions of *D. villosum* (TA2126, TA2197, TA10260, and TA10276) were individually pooled from five individual plants representing each accession. The sequences that represented Rusty and CS were trimmed to a length of 100 bp based on the mean sequence length for that run generated by the Ion Proton sequencing platform. The sequences for each *D. villosum* accession were also pooled and trimmed to 100 bp, and sequences with read depths of 4 to 9 were retained. The read depth cut-off is based on the trend of the frequency distribution of duplicated sequences. Similarly, each of the chromosomes from *D. villosum* additional lines (TA7677 to TA7683) was trimmed to a length of 100 bp and only sequences that had a read depth of 3 or greater were retained. Then, the sequences for all the *D. villosum* chromosome addition lines were pooled. In each case, if sequences less than 100 bp were detected posttrimming, these were excluded from downstream analysis.

From the pooled *D. villosum* addition line sequences, those with read depth greater than 1 were discarded, assuming that these were overrepresented by sequences in common with CS. Further, the remaining unique set of sequences in the *D. villosum* addition line pool were compared with the pooled CS sequences, and sequences that matched were discarded. This step ensured that sequences from CS that escaped the first filter on the *D. villosum* pool were removed. Next, sequences from the International Wheat Genome Sequencing Consortium (IWGSC) (The International Wheat Genome Sequencing Consortium 2014) that matched sequences in the CS filtered *D. villosum* pool were also discarded. To enable removal of IWGSC sequences, BLAST (Altschul et al. 1990; Madden 2002) was used, percentage identity was set to 100, and those with alignment length of 100 were removed from the *D. villosum* pool. The common sequences between CS and IWGSC filter *D. villosum* additional line pool and *D. villosum* parent pool with read depth of 4 to 9 were identified using an in-house R script.

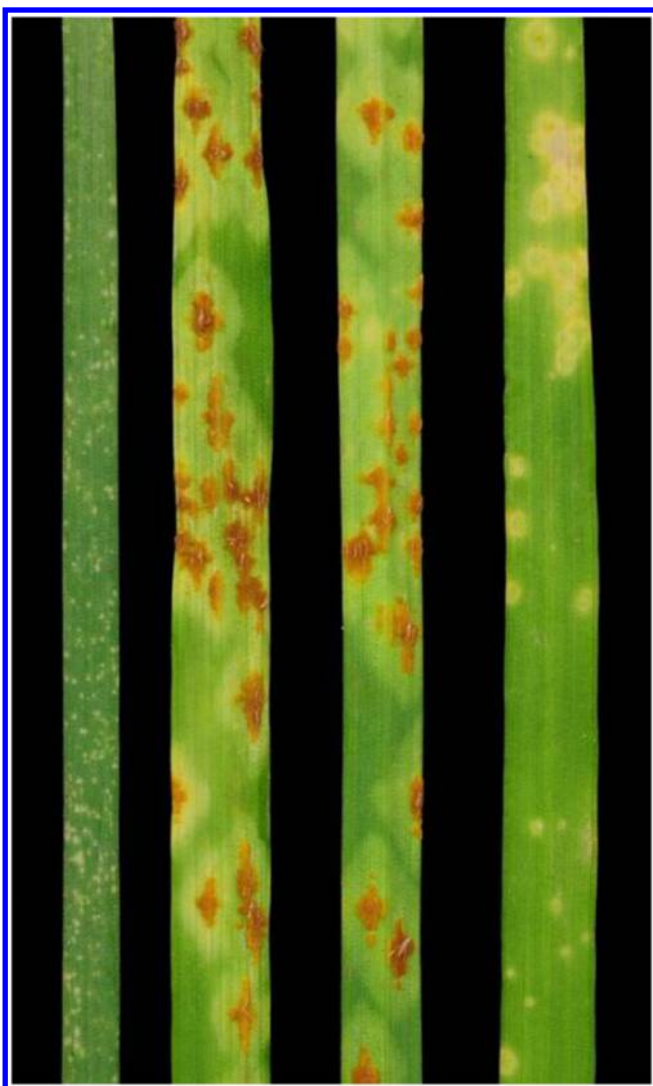


Fig. 1. Seedling reactions of parental lines and 10276-2V to *Puccinia graminis* f. *tritici* race TTKSK. Left to right: TA10276, Rusty, Chinese Spring, and 10276-2V.

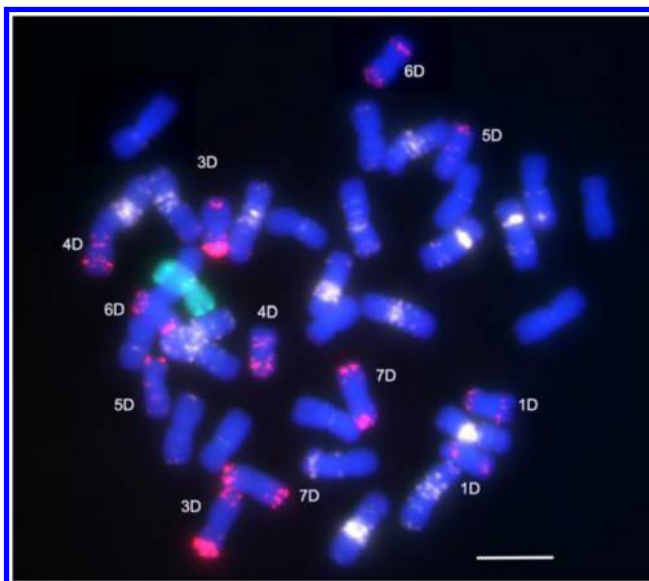


Fig. 2. Cytogenetic analysis of introgression line by combined genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH). A monosomic substitution line MS2V(2D) ($2n = 41$) 10276-2V is shown. Wheat chromosomes were identified by patterns of tandem repeats pAs1 (red) and (GAA)_n (white); *Dasypyrum villosum* chromatin was detected by GISH (green). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (blue). Bar corresponds to 10 μ m.

Results

Durum-*D. villosum* amphiploids for TTKSK screening. Four *D. villosum* accessions originating from the area surrounding the Mediterranean Sea were chosen to create durum-*D. villosum* amphiploids (Table 2). All four *D. villosum* accessions were highly resistant to infection against *P. graminis* f. *tritici* race TTKSK with 0; infection type (IT) (Supplementary Fig. S1). All of the amphiploids were also scored as resistant to TTKSK, with IT ratings ranging from 0; to 2, except one primary amphiploid of TA10276 with IT 3+. In cases where different primary amphiploids were tested from specific Rusty/*D. villosum* hybrids, responses to TTKSK infection were not always the same, indicating the heterogeneous nature of the *D. villosum* genome due to open pollination. Interestingly, in many cases, amphiploids were less resistant than *D. villosum* accessions. To examine the allelism of genes present among the accessions, an F_{2:3} amphiploid population Rusty/TA10276B//Rusty/TA2126B was created. All 120 families were resistant to TTKSK with IT 0;. This could be due to the presence of multiple effective genes in both TA10276 and TA2126, or that their resistance is based on the same or tightly linked loci.

Identifying *D. villosum* single-chromosome introgression lines with COS markers and rust screening. Twenty-four COS markers were initially selected because they showed polymorphic patterns between the wheat-*D. villosum* DA lines and CS and Rusty. In all cases, CS and Rusty had the same peaks, whereas single DA lines had unique peaks. Finally, one COS marker per *D. villosum* chromosome was selected (Table 3). COS marker genotyping was repeated for each generation until a representative set of single *D. villosum* introgression lines was recovered. A set of single TA10276-*D. villosum* chromosome introgression lines at BC₁F₂ to BC₄F₁ generations was recovered, except for chromosome 7V based on COS marker genotyping (Table 4). These lines were subjected to TTKSK screening and a line carrying 2V based on COS genotyping was highly resistant to TTKSK (Fig. 1). Self-pollinated progeny of the 2V introgression line showed a ;2 IT and the family was segregating for susceptibility. The 2V line was named 10276-2V. The *Sr* gene in 10276-2V was assigned temporary designation *SrTA10276-2V*. The remaining TA10276-*D. villosum* chromosome introgression lines were susceptible to TTKSK.

Cytogenetic analysis. The chromosome constitution of line 10276-2V was examined by a combined GISH and FISH method. We assumed that the missing wheat chromosomes were substituted by *D. villosum* homeologs. In 10 plants analyzed, chromosome numbers varied between 41 or 42; 1 or 2 complete *D. villosum* chromosomes, sometimes in combination with short- or long-arm telosomes or isochromosomes, were present, all wheat A- and B-genome chromosomes were detected, and one or two 2D chromosomes were absent. The plant phenotype and fertility can confirm whether an alien chromosome is compensating for a wheat homologue (Dvořák 1980; Friebe et al. 1993; Sears 1952). The phenotype of line 10276-2V confirmed that the *D. villosum* chromosome was compensating for wheat 2D. In addition, *D. villosum* chromosome arm-specific markers were used to verify the cytogenetic analysis. The line 10276-2V carried both long and short arms of 2V and no other *D. villosum* chromosome arms (Supplementary Table S1).

The monosomic substitution line MS2V(2D), carrying a single *D. villosum* chromosome 2V and missing wheat chromosome pair 2D with total chromosome number 41, is shown on Figure 2.

***D. villosum*-specific sequence marker development by GBS.** In order to facilitate further introgression of the *SrTA10276-2V* *D. villosum* resistance, chromosome-specific markers were developed via a GBS method. CS, Rusty, and wheat-*D. villosum* DA lines were sequenced and 177 million reads were generated from all of the samples with a mean read length of 159 bp. Initially, all seven wheat-*D. villosum* DA line GBS reads were combined and compared with CS GBS reads and the IWGSC CS draft genome sequence to remove the perfect-match reads, resulting in 111,000 unique reads. Subsequently, the remaining GBS reads were compared with four accessions of *D. villosum* to verify the specificity of the reads. The majority of the GBS reads occurred in the *D. villosum* accessions fewer than two times. These reads were then compared with each of the seven disomic wheat-*D. villosum* addition line GBS reads. In total, 9,369 reads were assigned to each *D. villosum* chromosome, with read frequency ranging from 1 to 26 times and an average of 3.9 reads per marker. After filtering redundant reads, 2,444 reads were identified as specific to *D. villosum* chromosomes, ranging from 132 to 840 markers per chromosome, with an average of 349 (Table 5; Supplementary File S1). Selected markers were verified for their specificity to *D. villosum* chromosomes by PCR (Supplementary Fig. S2).

Discussion

New *T. durum*-*D. villosum* amphiploids with TTKSK resistance were created. These amphiploids exhibited a range of phenotypes in response to TTKSK infection, indicating that there may likely be several stem rust resistance genes present, which are yet to be characterized in *D. villosum* germplasm. In addition, *D. villosum* is an open-pollinating species; therefore, the genomes are highly heterogeneous. In fact, when different primary amphiploids from the same *D. villosum* accession were inoculated with TTKSK, different disease responses were frequently observed. The only other *Sr* gene reported to have originated in *D. villosum*, *Sr52* on a T6AS·6V#3L translocation, is temperature sensitive, conferring a 2- infection type to TTKSK, and it is most effective at 16°C (Qi et al. 2011). In the current study, *SrTA10276-2V* was present on chromosome 2V and was derived from the *D. villosum* accession TA10276. Intercrossing of primary amphiploids of TA10276 and TA2126 showed no susceptibility, suggesting that these may carry the same gene. Alternative explanations are that they are tightly linked or several (4+) unlinked genes are segregating in the cross, assuming typical inheritance, and our population size was too small to recover susceptible progeny. The TTKSK infection type of *SrTA10276-2V* was ;2, which is distinct compared with the IT of *Sr52* under the same conditions.

COS markers developed based on rice-wheat orthologs (Quraishi et al. 2009) were used to genotype the wheat-*D. villosum* introgression lines. These COS markers were polymorphic between CS, Rusty, and wheat-*D. villosum* chromosomes. In our study, a combined analysis of GISH and FISH verified the COS genotyping result. According to Liu et al. (2011), DNA marker data and the GISH results generally agree; however, DNA marker genotyping

Table 5. Summary of *Dasyphyrum villosum* chromosome-specific genotyping-by-sequencing (GBS) reads

<i>D. villosum</i> chromosome	Total GBS reads	Total unique reads	Mean unique reads	Unique reads range
1V	504	132	3.8	1-22
2V	1,751	466	3.8	1-26
3V	3,113	840	3.7	1-26
4V	1,180	310	3.8	1-25
5V	652	144	4.5	1-18
6V	902	233	3.9	1-24
7V	1,267	319	4.0	1-18
Total	9,369	2,444	...	...
Overall mean	1,338.4	349.1	3.9	...

may create false-positive results by detecting chromatin in the absence of GISH signals. More dense marker systems may aid in discriminating between experimental error and the unusual but documented presence of cryptic translocation events (Tiwari et al. 2014). In addition, *D. villosum* is highly heterogeneous because of open pollination and suffers from rapid inbreeding depression, and the transferability of polymorphic markers from one accession to another accession is low. To address this, we tested four accessions of *D. villosum* for COS markers which can be used for each accession. Using these COS markers, we were able to recover a majority of single *D. villosum* chromosome introgression lines for each accession (data not shown).

Approximately 2,500 *D. villosum* chromosome-specific markers were detected by GBS. By testing these markers on Robertsonian translocation lines created by Liu et al. (2011), the markers can be further assigned to chromosome arms. This will help to minimize the number of markers for screening of introgression or mapping populations and these markers may be useful for marker-assisted breeding as translocation stocks with small introgression events are developed. Moreover, current *D. villosum* sequence information is limited, and there are only a few markers for each chromosome. Therefore, this marker system should streamline working with this allele-rich alien species.

Four *D. villosum* accessions were genotyped by GBS to allow investigation of polymorphism between accessions. Filtered GBS sequences that were only common among these four accessions were assigned to chromosomes. In our current study, we developed presence or absence variation (PAV) markers that are specific to individual *D. villosum* chromosomes. However, the GBS raw dataset is dynamic and can be reanalyzed to uncover further information (Poland and Rife 2012). Therefore, the same raw dataset can be used as is and integrated with new data to develop SNP or PAV markers in future studies.

The 10276-2V line was determined to be a monosomic substitution line MS2V(2D) using cytogenetic analysis. It was missing a pair of 2D chromosomes and one *D. villosum* chromosome 2V. Chromosome 2V from various *D. villosum* accessions has been previously associated with resistance to eyespot (caused by *Oculimaculata yallundae* and *O. acufomis*), Wheat streak mosaic virus resistance, stripe rust (caused by *P. striiformis* f. *tritici*), and zinc accumulation (Chen et al. 2008; De Pace et al. 2011). The MS2V(2D) substitution line is the starting point for further directed chromosome engineering aimed at producing compensating wheat-2V recombinants with shortened *D. villosum* chromatin but still retaining the stem rust resistance gene.

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