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Targeted Sequencing of the Short Arm of Chromosome 6V of a Wheat Relative *Haynaldia villosa* for Marker Development and Gene Mining

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Abstract: The short arm of chromosome 6V (6VS) of *Haynaldia villosa* has been used in wheat breeding programs to introduce *Pm21* resistance gene against powdery mildew (Pm) and some other genes. In this this study, 6VS was flow-sorted from wheat-*H. villosa* ditelosomic addition line Dt6VS and sequenced by Illumina technology. An assembly of 230.39 Mb was built with contig N50 of 9.788 bp. In total, 3.276 high-confidence genes were annotated and supported by RNA sequencing data. Repetitive elements represented 74.91% of the 6VS assembly. The 6VS homologous genes were identified on homologous group 6 in six Triticeae species confirming their synteny relationships. Out of 45 NB-ARC domain proteins identified on 6VS, 15 were upregulated and might also be involved in the innate immunity of *H. villosa* to Pm. High thousand grain weight (TGW) for 6VS/6AL translocation line was not attributable to *GW2-6V* gene. Based on the intron size differences, 119 intron-target (IT) markers were developed to trace the 6VS chromatins introduced into wheat background. The assembled 6VS genome sequence and the developed 6VS specific IT markers in this work will facilitate the gene mining and utilization of agronomic important genes on 6VS.

Keywords: comparative genome analysis; chromosome sorting; genome sequencing; marker development; flow cytometry; *Haynaldia villosa*

Citation: Zhang, X.; Wan, W.; Li, M.; Yu, Z.; Liu, J.; Holušová, K.; Vrána, J.; Doležel, J.; Wu, Y.; Wang, H.; et al. Targeted Sequencing of the Short Arm of Chromosome 6V of a Wheat Relative *Haynaldia villosa* for Marker Development and Gene Mining. *Agronomy* **2021**, *11*, 1695. https://doi.org/10.3390/agronomy11091695

Academic Editor: Vojtech Holubec

Received: 22 June 2021
Accepted: 23 August 2021
Published: 25 August 2021

1. Introduction

Haynaldia villosa L. (2*n* = 14, genome VV), a wild relative of common wheat (*Triticum aestivum* L.), carries resistance genes to several wheat diseases, including powdery mildew, wheat yellow mosaic virus, eyespot, take-all and rusts [1]. It has also been credited for improving tiller number [2,3], high grain protein content [4–6], and tolerances to frost and drought [7,8]. These characters make *H. villosa* a highly attractive source of important genes and alleles for wheat improvement [1]. In previous studies, several useful genes were mapped on short arm of chromosome 6V, such as the *Pm21* locus, which provides immunity or high resistance to all powdery mildew isolates, and *NAM-V1*, which contributes to increased grain protein content (GPC) in the wheat-*H. villosa* 6AL/6VS translocation lines [9,10]. The availability of genome sequence will greatly accelerate further gene mining in *H. villosa* and effective alien gene introduction and utilization in wheat breeding while minimizing the introduction of unfavorable alien chromatins.

The progress in DNA sequencing technology, i.e., whole genome shotgun technology makes the production of whole genome sequence assemblies feasible, especially for species with huge and complex genomes such as wheat. The number of sequenced genomes of wheat and relatives has increased recently [11–13]. However, if chromosomal locations of target genes in a certain genotype are known, the most effective option is to sequence only the chromosome or chromosome arm of interest. The approach of dissection of the genome into its component chromosomes and sequencing only the targeted chromosomes significantly reduce the project costs. Sequencing chromosomes from multiple lines of a species for comparative genomic study could also be accomplished through this method [14]. It simplifies bioinformatic analyses due to reduced volume of sequence data.

Targeted sequencing of a particular chromosome is possible after isolating a required number of chromosomes by flow cytometric sorting [15–22]. Next-generation sequencing of flow-sorted chromosomes has been used to develop molecular markers in *Ae. geniculata* and *H. villosa* [23–25]. Importantly, sequencing DNA from flow-sorted chromosomes facilitated the production of draft genome assemblies of barley [26], rye [27] and common wheat [28] and to isolate genes in wheat and barley either by the MutChromSeq strategy [29] or the TACCA approach [30].

Purification of a particular chromosome by flow sorting may be hampered by the inability to discriminate the chromosome from other chromosomes by karyotype analysis if its size or relative DNA content has no obvious difference. Various strategies have been developed to overcome this difficulty, and one of them is to sort translocation or deletion chromosomes with altered size [16–20]. Larger deletions are not viable in diploids, but they may be developed from wild type chromosomes after they are introduced to a polyploid species, such as wheat, which tolerates aneuploidy. Using this approach, Tiwari et al. sorted chromosome 5 Mg from a wheat/*Ae. geniculata* disomic substitution line [21]. Similarly, Xiao et al. used a wheat-alien ditelosomic addition line "NAU1201" to isolate chromosome arm 4VS of *H. villosa* [22]. The creation of lines for targeted chromosomes or chromosome arms is therefore a crucial step for this flow cytometric method. These valuable recourses could already be available in some cases, thus tremendously facilitate the adoption of the method.

In this work, 6VS of *H. villosa* was flow-sorted from a *T. aestivum–H. villosa* ditelosomic addition line containing a pair of short arms of chromosome 6V. The isolated 6VS was sequenced and reads were assembled. The obtained draft sequence was used to characterize the molecular composition of 6VS including repeat DNA content, identify genes and comparative genome analysis with *T. aestivum* species and other sequenced grass species. The 6VS assembly was also used to develop 6VS-specific markers to support alien introgression breeding of wheat and the cloning of favorable genes from 6VS.

2. Materials and Methods

2.1. Plant Materials

H. villosa (VV, 2*n* = 14, Accession No. 91C43) was obtained from Cambridge Botanical Garden, UK. The other three accessions of *H. villosa* were kindly provided by Germplasm Resources Information Network (GRIN) (*H. villosa*#1: PI251478, *H. villosa*#2: PI491576, *H. villosa*#3: PI598391). The *T. aestivum–H. villosa* ditelosomic addition line Dt6VS [2*n* = 42(AABBDD) + 2t(6VS)] (Accession No. NAU1202), three *T. aestivum–H. villosa* small fragment translocation lines (Accession No. NAU418, NAU419 and NAU1203), and *T. aestivum–H. villosa* T6VS·6AL translocation line 92R137 (Accession No. NAU405) were developed at the Cytogenetics Institute, Nanjing Agricultural University (CINAU, hereafter) [31,32]. Three additional lines of *H. villosa* chromosome 6V added to wheat (DA6V#1, DA6V#3, DA6V#4) were identified by Qi et. al. and maintained at CINAU [33]. Common wheat (*T. aestivum*, AABBDD) cv. Chinese Spring maintained at CINAU was used as a control in this work.

2.2. Chromosome Sorting and DNA Sequencing

Suspensions of mitotic metaphase chromosomes were prepared from synchronized meristem root tips of young seedlings according to Vrána et al. and Kubaláková et al. [34,35]. GAA microsatellite repeats on isolated chromosomes were fluorescently labeled by FISHIS [36] using GAA-fluorescein isothiocyanate (FITC) conjugate (Sigma, Saint Louis, MO, USA) and counterstained by DAPI (4′,6-diamidino-2-phenylindole) at 2 µg/mL. The samples were analyzed by FACSAria II SORP flow cytometer and sorter (Becton Dickinson Immunocytometry Systems, San José, CA, USA) at rates of 2000–3000 particles per second and sort windows were set on bivariate flow karyotypes FITC vs. DAPI fluorescence. The identity of sorted particles and contamination of sorted fractions by other chromosomes were determined following Kubaláková et al. [37]. Briefly, one thousand particles were sorted from each sample into a 7 µL drop of P5 buffer on a microscope slide. After air-drying, the slides were used for FISH with probes of pSc119.2 and Afa family repetitive DNA sequences to verify the sorted 6VS chromosome arm and evaluated by fluorescence microscopy [38].

Chromosomes were sorted at rates of 15–20/sec into 40 µL sterile deionized water in 0.5 mL PCR tubes and two different 6VS DNA samples were prepared and sequenced. The first was produced by multiple displacement amplification (MDA) of DNA prepared from two batches of 100,000 copies of 6VS telosomes. The amplification was done using Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Piscataway, NJ, USA) as described by Šimková et al. [39] and the two MDA products were pooled into one sample to reduce amplification bias. Two micrograms of amplified DNA were used to prepare sequencing library using TruSeq DNA PCR-Free Library Prep Kit (Illumina, San Diego, CA, USA). The library was sequenced in one run on Illumina MiSeq System (1000 bp insert, 2 × 300 bp) yielding 14 Gb sequence data (~44× coverage of 6VS). The second type of 6VS DNA sample was not amplified and DNA from 100,000 copies of 6VS telosome was purified and directly used to prepare sequencing library using Nextera DNA Library Prep Kit (Illumina). The library was sequenced in one run on Illumina MiSeq System (500 bp insert, 2 × 300 bp) yielding 10.2 Gb sequence data (~32× coverage of 6VS). The sequenced reads data of this research were available in NCBI (PRJNA590539). Four k-mer sizes (41, 45, 49, and 63) were used to de novo assemble the raw data using the software of Hecate (http://bgi-international.com/us/, accessed on 18 January 2019). The k-mer sizes which generated the assembly with the best sequence coverage and N50 size were finally selected.

2.3. Identification of Repetitive Sequences

The repetitive DNA regions of 6VS assembled sequence was identified and masked using the software of RepeatMasker (http://www.repeatmasker.org/, accessed on 25 February 2019). Two repeat libraries, TREP database and Repbase Update, were used to search the repetitive sequences of the 6VS with the default settings.

2.4. Transcriptome Data

The 6VS/6AL translocation line was grown in a growth chamber with 20 °C/16 °C temperatures (day/night), and 16 h/8 h of light/dark. The translocation line was inoculated with Bgt isolates E26 and E31 at two-leaf stage, together with water treatment as control. Samples were collected at 3 and 24 h after Bgt and water inoculation, respectively, followed by freezing in liquid nitrogen for subsequent RNA extraction. The samples were submitted to the BGI for sequencing using the Illumina Hiseq 4000 platform. Raw sequences were filtered by removing those containing ploy-N, poor-quality reads, and the adapters to obtain high-quality data for further analysis. De novo transcriptome assembly was built using Trinity software [40]. A total of 107,797 unigenes were obtained after redundancy removal of the result of Trinity. The clean reads were mapped to unigenes using Bowtie2, and then calculated for gene expression levels with RSEM [41,42]. *Bgt* isolates E26 and E31 were obtained from Institute of Plant Protection, Chinese Academy of Agricultural Sciences.

2.5. Identification of Coding Sequences

The gene prediction of repeat-masked 6VS sequences was performed through AUGUSTUS program. The transcriptome data of *H. villosa*, which contain 204,258 unigenes, was used to provide evidence of the loci with coding genes. The predicted genes were BLASTn against the transcriptome data to define the evidenced gene, with more than 95% identity and at least 300 bp coverage on a unigene of transcriptome. For GO analysis of predicted genes, Blast2GO and WEGO software were performed to get GO annotation and GO functional classification, respectively.

2.6. Development of Intron Target Markers

Firstly, we extracted the annotated coding sequence (CDS) of the unigenes from the two gene databases of *Ae. tauschii* chromosome 6DS and *T. aestivum* chromosome 6DS. Then, all genes were compared with the genomic sequences of Chinese spring short arm chromosomes of group 6 and *H. villosa* 6VS through BLASTn program. The genes which have homologous copies and predicted at least one intron among 6DS, 6BS, 6AS and 6VS chromosomes were selected. Thirdly, we determined and compared the intron sizes of selected genes, and chose the target introns to design the primer pairs with predicted amplification sizes in 6VS differed from 6DS, 6BS and 6AS simultaneously at least 10%. Primer 3 (https://bioinfo.ut.ee/primer3-0.4.0/, accessed on 20 August 2019) was used to design primers in the exons that flanking the target introns.

3. Results

3.1. Flow Sorting and Sequencing of Chromosome Arm 6VS of H. villosa

Flow cytometric analysis of chromosomes isolated from *T. aestivum–H. villosa* 6VS ditelosomic addition line resulted in bivariate flow karyotypes FITC (log scale) vs. DAPI (linear scale) fluorescence, on which a number of populations could be resolved (Figure 1). The population representing 6VS telosome was identified after screening all populations with lower DAPI fluorescence, which were expected to correspond to smaller chromosomes. Microscopic analysis of flow-sorted particles after FISH with probes for pSc119.2 and Afa family repeats enabled unambiguous identification of the population representing 6VS telosomes (Figure 1). A detailed microscopic analysis showed that 6VS telosome could be sorted at an average purity of 89.41% [14]. The sorted DNA was amplified by multiple displacement amplification (MDA) reactions before Illumina sequencing. Sequencing of DNA amplified from flow-sorted chromosome 6VS in Illumina MiSeq system generated 47.7 Gb high-quality paired-end reads from two libraries, with insert sizes of 500 bp and 1000 bp, respectively.

After assembly using Hecate software, a total of 230.39 Mb draft sequences was obtained. The sequences consisted of 153,177 scaffolds. The maximum and minimum lengths of the scaffolds were 138,620 bp and 100 bp, respectively. The contig N50 and mean scaffold length were 9.788 bp and 1.464 bp, respectively (Table 1).

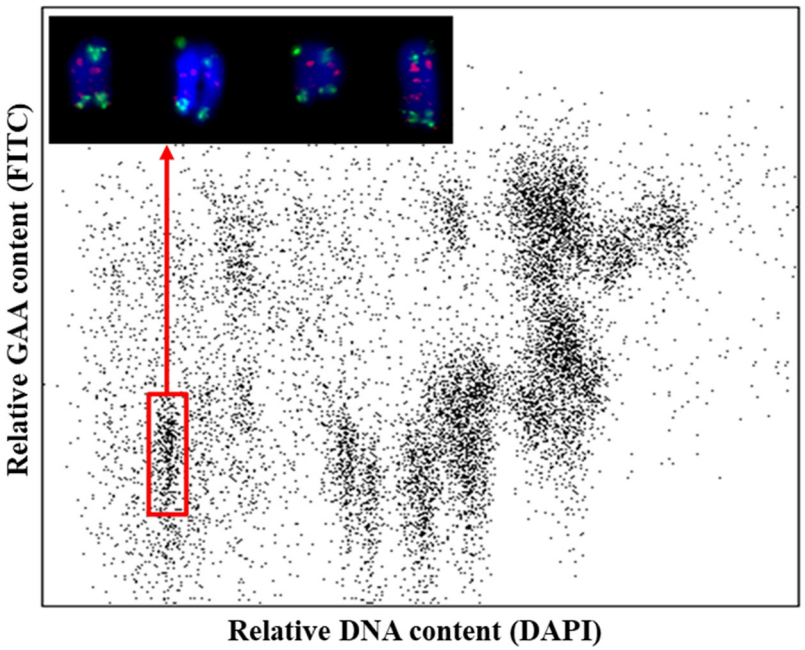

Figure 1. Flow sorting of *H. villosa* chromosome arm 6VS. Bivariate flow karyotype obtained after the analysis chromosomes isolated from bread wheat-*Haynaldia villosa* 6VS ditelosomic addition line. Prior to analysis, GAA microsatellite repeats on chromosomes were labeled by fluorescein isothiocyanate (FITC) and counterstained by 4′,6-diamidino-2-phenylindole (DAPI). The population representing 6VS telosome is marked by a red rectangle. The inset shows examples of flow-sorted 6VS telosomes after fluorescence *in situ* hybridization (FISH) with probes for pSc119.2 (yellow-green) and Afa family repeats (red).

Table 1. The statistics of assembly of flow-sorted short arm of *H. villosa 6V* chromosome.

Total bases (Gbp)	47.7
Number of assembly scaffolds	153,177
Total assembly bases (bp)	230,388,792
Max. length of assembly scaffolds (bp)	138,620
Min. length of assembly scaffolds (bp)	100
N50 (bp)	9.788
Mean length (bp)	1.464
GC-content (%)	45.68

3.2. The Repetitive DNA Elements in the 6VS Sequence

Using RepeatMasker software, a total of 181.29 Mb out of 230.39 Mb 6VS assembly was identified as repetitive sequences, which accounted for 78.31% (Table 2). Among the repeat elements, the most abundant were LTR retrotransposons comprising of 64.94%, out of which 51.91% were *Gypsy* superfamily repeats, followed by 10.81% of *Copia* superfamily. DNA transposons were mainly represented by *TIR* family, which made up 6.53% of all repeats. After masking all repetitive DNA elements, the remaining nonrepetitive sequence reads from 6VS equaled 49.1 Mb, which was used for the following gene prediction and sequence comparisons.

Table 2. Identification of repetitive DNA elements in short arm of *H. villosa* 6V chromosome.

Type	Subtype	Total Length (bp)	% Genome
DNA transposon			
	TIR	11,269,751	6.53
	Helitron	189,843	0.11
retrotransposon			
	LTR_*Copia*	18,656,357	10.81
	LTR_*Gypsy*	89,588,481	51.91
	LTR_Unknown	3,831,370	2.22
	SINE	1,691,326	0.98
	Unknown	4,694,291	2.72
tandem repeat		535,011	0.31
unknown		4,694,291	2.72

3.3. Gene Content of Chromosome Arm 6VS

By ab initio gene prediction using AUGUSTUS software, 5.973 predicted coding genes were preliminarily identified from repeat-masked scaffold of 6VS. Referring to the transcriptome data of *H. villosa* [21] as the evidence of coding loci, 3.276 genes on 2.871 scaffolds of 6VS were retained and deemed as high-confidence genes (HCG). The gene length distribution is shown in Figure S1A. The genic sequences represented a total length of 5,278,412 bp, which accounted for 2.3% of the 6VS assembly. Totally, 1.672 genes were classified to one or more Gene Ontology (GO) terms (Figure S1B). The number of genes which was annotated into biological process, molecular function and cellular component were 1.432, 1.150 and 1.441, respectively.

In order to test the quality of the 6VS annotation, genes *NLR-V* [43], *STPK-V* [31] and *NAM-V1* [9] which have been cloned from 6VS were selected for BLASTn search. The sequence homology of the cloned genes and the annotated sequences were 99.93%, 100.00% and 99.93%, respectively, implying a high quality of the *H. villosa* 6VS annotation. As *H. villosa* and *H. vulgare* are evolutionarily closely related, we performed micro-collinear analysis between two randomly selected scaffolds (scaffold6533, scaffold16282) of *H. villosa* and *H. vulgare* genome. Sixteen and six annotated genes on scaffold6533 and scaffold16282, corresponded to 14 and six genes of *H. vulgare* in their collinear region, respectively (Figure S2), further indicating the reliability of 6VS annotation. Thus, the 6VS draft sequence obtained in this work will facilitate further extensive gene mining from 6VS.

3.4. Comparative Genome Analysis of 6VS Sequence Composition

By referring to the genomic reference of common wheat cv. Chinese Spring released by IWGSC [43], the HCGs of 6VS were used to identify the syntenic regions of 6VS on wheat chromosomes 6A, 6B and 6D. By referring to the released genome sequences of other *T. aestivum* species, the 6VS syntenic regions were identified on chromosomes 6A and 6B of tetraploid *T. dicoccoides*, 6D of *Ae. tauschii*, 6A of *T. urartu* and 6H of *H. vulgare*. After filtering, 2.867 6VS HCGs had 1.499, 1.577 and 1.430 blastn hits with their homologous genes in wheat chromosomes 6A, 6B and 6D, respectively; 1.323 and 1.374 blastn hits with their homologous genes in *T. dicoccoides* chromosomes 6A and 6B, respectively; 1.301, 1.424 and 1.307 blastn hits with their homologous genes in *Ae. tauschii* chromosome 6D, in *T. urartu* 6A and in *H. vulgare* 6H, respectively. Moreover, 634 out of 2.867 genes were shared by all eight genomes. The syntenic genes on *T. aestivum* 6A, 6B and 6D, *T. dicoccoides* 6A and 6B, *Ae. tauschii* 6D, *T. urartu* 6A and *H. vulgare* 6H were plotted on chromosomes to highlight their syntenic regions, according to their physical position (Figure 2). As expected, the syntenic regions with high gene density were observed on chromosome arms 6AS, 6BS and 6DS of *T. aestivum*, 6AS and 6BS of *T. dicoccoides*, 6DS of *Ae. tauschii*, 6AS of *T. urartu* and 6HS of *H. vulgare*.

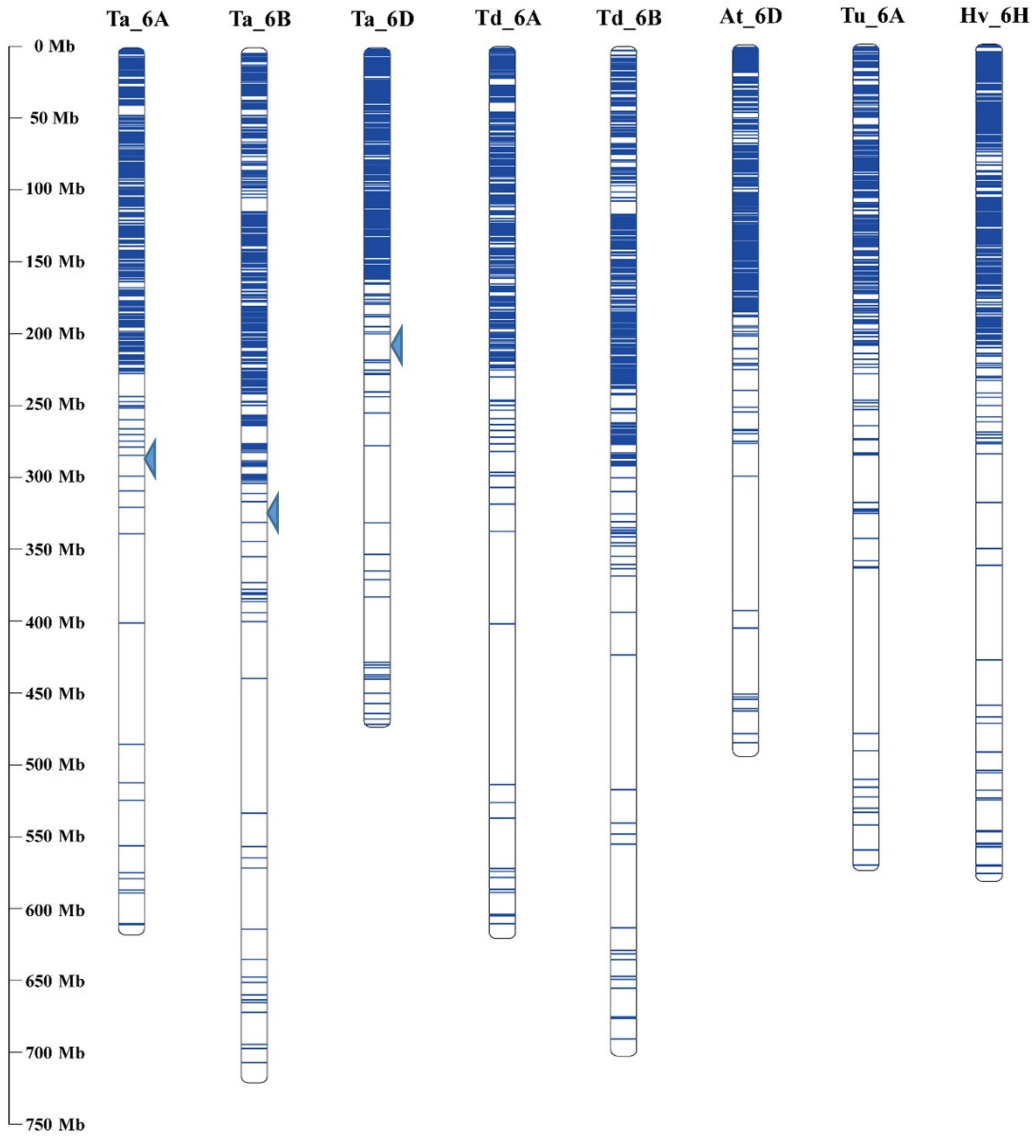

Figure 2. Comparative analysis of 6VS DNA sequence. The distinguish of 6VS homologous genes in group 6 of Triticeae genomes. The blue lines represent the homologous genes of 6VS located on the chromosomes of Triticeae genome. Ta, Td, At, Tu and Hv represent *T. aestivum*, *T. dicoccoides*, *Ae. tauschii*, *T. urartu* and *H. vulgare*, respectively. The blue arrows represent the position of the centromere of common wheat.

3.5. NB-ARC Domain Proteins Enrichment and their Expression Profiling after Bgt Infection

In China, the translocation line T6VS·6AL has been used as a backbone parent of wheat breeding, and more than 20 commercial varieties carrying the chromosome have been cultivated for powdery mildew resistance. Despite *NLR1-V* for *Pm21* has been cloned, a question comes out whether only one gene could persist resistance for 30 years. Here, we identified resistance gene analogs (RGAs) from 6VS assembly and preliminarily investigated their expression profiling after infection of *Blumeria graminerum f. sp tritici* (*Bgt*). NB-ARC domain proteins are a typical type of RGAs. In the 6VS assembly, a total of 45 genes were predicted to encode NB-ARC domain proteins using HMMER model [44]. We analyzed transcriptome of the wheat-*H. villosa* T6VS/6AL translocation line after the treatment with two *Bgt* isolates E26 and E31. We found that, of the 45 genes, 28 were expressed after inoculation of both isolates within 24 h, with 15 genes up-regulated two-fold or more when compared to the control (Figure 3). As 6VS chromatin introduced to wheat showed the main contributor of the resistance to various *Bgt* isolates, we propose

that apart from the *NLR1-V* and *STPK-V* cloned from 6VS, there might exist other disease resistance encoding genes involved in the innate immunity of *H. villosa* to *Bgt* infection. Functional validation of these *R* genes in resistance to powdery mildew will need to be carried out in the future.

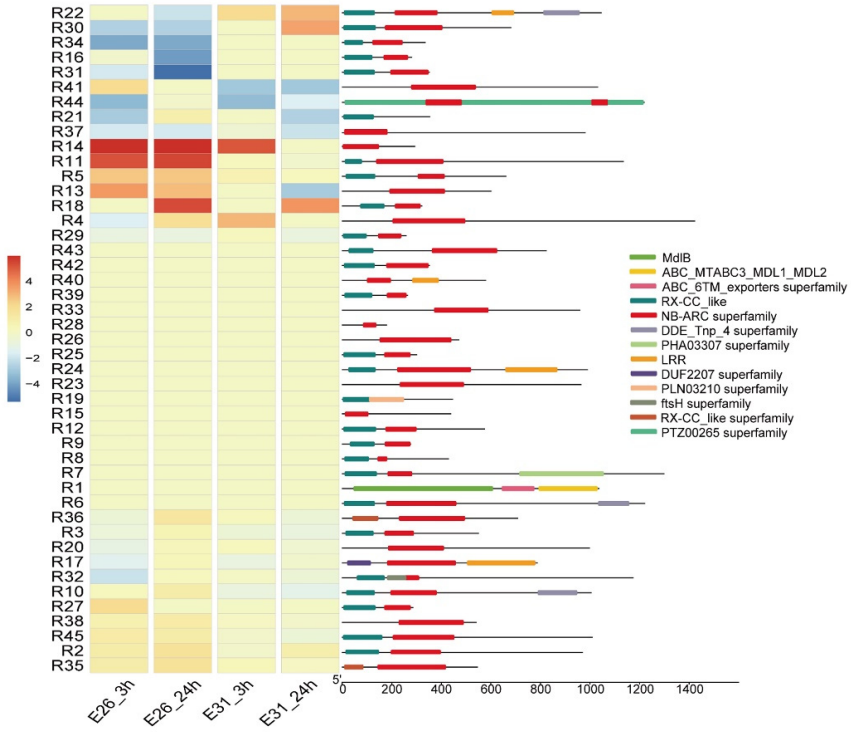

Figure 3. Gene expression patterns in response to powdery mildew infection and gene structures of the resistance gene analogs (RGAs) from 6VS of *H. villosa*. Left: The heatmap for the expression of predicted RGAs that treated with two *Bgt* isolates, E26 and E31, respectively at 3 and 24 h after inoculation. Right: The structure of resistance gene analogs which predicted from 6VS of *H. villosa*. Different colors represent different domains. The scale represents the length of amino acids of genes.

3.6. Haplotype Analysis of GW2-6V

Four SNPs that occurred in the promotor region of *TaGW2-6A* were reported to be associated with thousand-grain weight (TGW) at positions −998 bp, −739 bp, −593 bp and −494 bp, in which SNP at −494 bp showing significant association with TGW and located in the 'CGCG' motif [45]. SNP-494 has most effect on *TaGW2-6A* expression level and TGW, with haplotypes of the A allele having significantly lower *TaGW2-6A* expression higher TGW compared with those with the G allele. To figure out if the increased TGW of 6VS/6AL translocations was due to the substitution of 6AS with 6VS, the *TaGW2-6A* gene homologue *GW2-6V* was identified in the 6VS assembly. However, the *GW2-6V* in *H. villosa* belongs to G allele at SNP-494and was associated with low TGW (Figure 4), implying *GW2-6V* allele was not attributable to high TGW.

	-988	-739	-593	-494	
HvGW2-6V -promotor	AAG**G**CG.....	CCCGTGCA**G**TAGCT.....	TCG**G**AC.....	CC**G**CGG	High TGW
TaGW2-6A-promotor Hap_1	AAG**G**CG.....	CCCGTACA**G**CAGCT.....	TCG**G**AC.....	CC**G**CGG	Low TGW
TaGW2-6A-promotor Hap_2	AAG**G**CG.....	CCCGTACA**G**CAGCT.....	TCG**A**AC.....	CC**G**CGG	Low TGW
TaGW2-6A-promotor Hap_3	AAG**G**CG.....	CCCGTACA**A**CAGCT.....	TCG**G**AC.....	CC**G**CGG	Low TGW
TaGW2-6A-promotor Hap_4	AAG**A**CG.....	CCCGTACA**G**CAGCT.....	TCG**A**AC.....	CC**G**CGG	Low TGW
TaGW2-6A-promotor Hap_5	AAG**G**CG.....	CCCGTACA**A**CAGCT.....	TCG**G**AC.....	CC**A**CGG	High TGW

Figure 4. The SNPs at the promotor region of *TaGW2-6A* and *GW2-6V*. Characters that were highlighted with red color were the SNPs that reported association with thousands of grain weight (TGW) within the promoter region of *TaGW2-6A* and *GW2-6V*. 'CGCG' motif was underlined.

3.7. Development of 6VS Specific Intron Targeted (IT) Markers

Zhang et al. and Wang et al. developed IT markers for all chromosomes except for 6VS of *H. villosa* [23,24]. With the shotgun sequences of 6VS, IT markers for this short arm could now be developed. All 2,063 annotated genes from *Ae. tauschii* 6DS were aligned against the wheat genome reference and 6VS assembly sequences, and exon-exon junction lengths on chromosomes 6AS, 6BS, 6DS of *T. aestivum* as well as on 6VS were determined. A total of 222 genes had the intron length in *H. villosa* differing by at least 10% as compared to those in wheat subgenomes A, B and D. Then, we designed PCR primers in the conserved exon regions which flanking the targeted introns using Primer 3 software (http://frodo.wi.mit.edu/primer3/, accessed on 20 August 2019).

The designed 222 IT primers on 6VS of *H. villosa* were tested by PCR analysis using DNA samples of *T. aestivum* cv. Chinese Spring (AABBDD), *H. villosa* (VV) and *T. aestivum–H. villosa* T6AL·6VS translocation line. If the primer pair amplifies a distinct PCR product visualized only in *H. villosa*, and *T. aestivum–H. villosa* T6AL·6VS translocation line while not in common wheat, it was considered 6VS-specific marker. In total, 119 6VS-specific markers were obtained with a success rate of 53.60% (Table S1). All these markers were tested on six lines including three other lines of *H. villosa* and three different addition lines for *H. villosa* 6V for the detection of variation within the species. Except for seven markers that could not detect polymorphism between common wheat c.v. Chinese Spring and part of above lines (number 1–4), the remaining 112 markers could be used to trace 6VS alien chromatin across the different *H. villosa* lines (Figure S3, Table S2).

All IT markers were further tested using three different translocation lines, NAU418, NAU419 and NAU1203, all involving 6VS but having different introgressed 6V segments. The chromosome arm could be dissected into four bins: bin1 to bin4 (Figure 5), which contained 34, 11, 46 and 29 markers, respectively. Given that all three translocation lines are resistant, consistent with previous results, the powdery mildew resistant gene *Pm21* was mapped into bin3. The 40 markers within this physical bin are suitable for marker-assisted breeding.

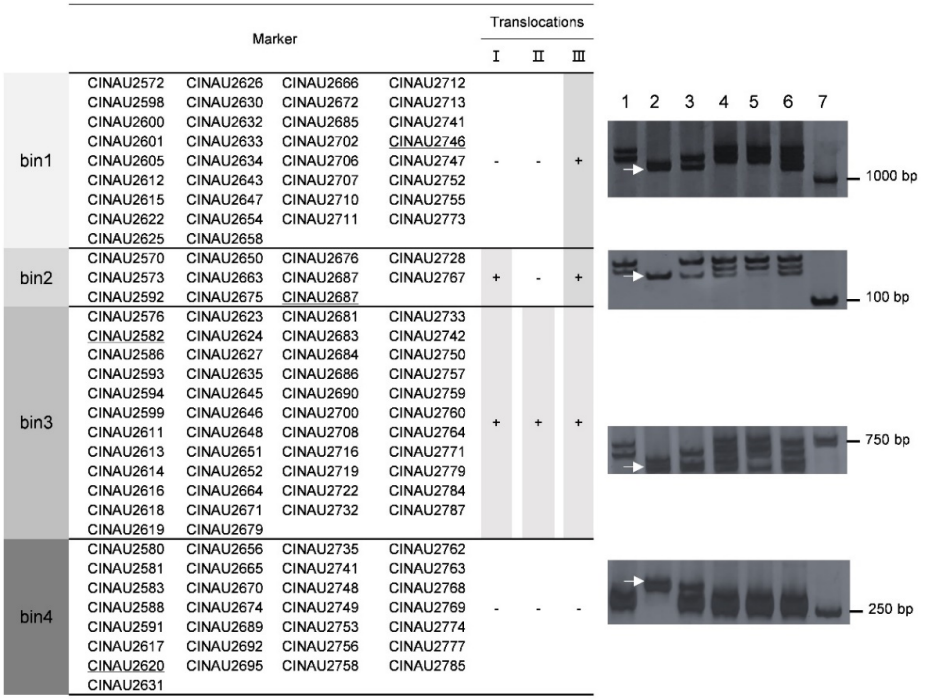

Figure 5. Development of IT markers and construction of a cytogenetic physical map of *H. villosa* 6VS chromosome arm. Left: Molecular markers in each bin. Right: The amplification results of representative markers in each bin which was underlined in the left. 1: *T. aestivum* (AABBDD); 2: *H. villosa*

(VV); 3: *T. aestivum–H. villosa* translocation line (T6VS·6AL); 4–6: Three *T. aestivum–H. villosa* small fragment translocation lines (NAU418, NAU419 and NAU1203); 7: DNA Ladder.

4. Discussion

4.1. Aneuploid Germplasm Facilitates Flow-Sorting Target Chromosomes or Chromosome Arms

In order to characterize the short arm of *H. villosa* chromosome 6V (6VS) at DNA level, we combined flow cytometric chromosome sorting and next-generation DNA sequencing. When compared to whole genome sequencing, this approach provided a massive and lossless reduction of DNA sample complexity and facilitated DNA sequence analysis. A chromosome can be purified by flow sorting if it differs in relative DNA content from other chromosomes in a karyotype, which is not the case of chromosome 6V in *H. villosa*. Thus, we used *T. aestivum–H. villosa* 6VS ditelosomic addition line to sort 6VS, since the telocentric chromosome 6VS is smaller in size compared with other intact chromosomes. With the aim to achieve a high resolution of 6VS, we employed bivariate analysis of DNA content (DAPI fluorescence) and the amount of GAA microsatellites labeled by FITC following the FISHIS protocol [36]. This approach permitted sorting 6VS arm at almost 90% purity.

4.2. The Available 6VS Sequences Would Facilitate the Introduction of Interest Genes with Minimized Linkage Drag by Chromosome Engineering

H. villosa has been an important donor of disease resistance in wheat breeding, and *Pm21* transferred from *H. villosa* into wheat remains the most effective powdery mildew resistance gene [10]. *Pm21* transferred from wheat–*H. villosa* translocation line T6AL·6VS, has been successfully utilized in wheat breeding, and more than 20 wheat varieties carrying the 6VS·6AL translocation chromosome have been released in China [46]. Although *Pm21* has been cloned, its introduction by genetic transformation may not be acceptable by the market [47]. Thus, the introgression of alien chromatin harboring traits of interest by chromosome engineering remains a priority. However, due to linkage drag, this strategy often introduces favorable traits together with deleterious loci, such as compromised yield or quality [48]. Thus, advanced chromosome engineering is needed to minimize alien chromatin during alien introgression breeding. The main procedures for reducing alien chromatin in wheat is to induce chromatin break-rejoining by ionizing radiation, or induce meiotic recombination between the alien chromatin and its homoeologous common wheat counterpart. The available of 6VS genome sequence enables us to gain the knowledge of beneficial and deleterious gene alleles, if these homoeologous genes were extensively studied, such as *GW2-6V*, which contributed to low TGW. Therefore, people could define the size of introgressed chromatin to preserve as many beneficial genes and remove as many deleterious loci as possible.

4.3. The High TGW for 6VS/6AL Translocation Line Was Not Attributable to GW2-6V

Wheat cultivars carrying the 6VS/6AL translocation have been used extensively in wheat production, accumulating acreage, now being more than four million hectares in China [10]. The translocation lines improved not only high powdery mildew resistance, but also an increase of TGW [49]. In a previous study, *TaGW2-6A* was described as a negative regulator of grain-width and grain-weight [45,50,51]. The higher expression of *TaGW2-6A* was associated with lower TGW and vice versa. The SNP-494 in 'CGCG' motif of the promoter region was found to underlie the trait, of which SNP-494_G (CGCG motif) haplotype has higher expression and lower TGW, and SNP-494_A (CACG motif) haplotype has lower expression but higher TGW [45]. From our 6VS genomic sequence, GW2-6V belongs to SNP-494_G haplotype. We speculate that higher TGW of the 6VS/6AL translocations might be affected by other genes rather than *GW2-6V*, or that the expression of alien gene is suppressed due to genomic shock in wheat background although the genotype at position −494 was the same with low TGW.

4.4. Development of Specific Molecular Markers Using Chromosome Sorting Strategy

The development of molecular markers is now much easier than before due to falling costs of next-generation sequencing. As shown in this work, this is true also in species without genome sequence, especially if a chromosome of interest can be purified by flow sorting. The sequences from alien chromosomes could then be combined with available wheat genome sequences to develop molecular markers suitable for detecting alien chromatin. Tiwari et al. developed 2.178 5MgS-specific SNPs for *Ae. geniculata* by combining chromosome flow sorting and sequencing and highlighted the power of this approach for mining markers specific for alien chromatin [25]. Zhang et al. developed 1,624 intron targeting markers for all *H. villosa* chromosomes, except 4VS and 6VS arms, and out of them, 841 (51.79%) markers were specific for tracing *H. villosa* chromatin in wheat background [23]. Wang et al. developed 359 intron targeting primers by combining chromosome sorting and sequencing, among which 232 (64.62%) can be used to trace the 4VS chromatin in the wheat background [24]. In this study, with the availability of the 6VS sequence, we designed 222 IT primer pairs and 119 (53.60%) were proved to be 6VS-specific. Apart from improving the knowledge of genome structure of an important donor of genes in wheat improvement and development of markers to support its use in alien introgression breeding of wheat, the results of this work confirm that chromosome sorting combined with next-generation sequencing is an efficient strategy for IT marker development.

5. Conclusions

Here, we report a draft DNA sequence of *H. villosa* chromosome arm 6VS and annotation of high-confidence of 3.276 genes. The coding genes showed a fine synteny with Triticeae group 6 chromosomes. A total of 119 IT markers specific to 6VS were developed and used to identify 6VS chromatin in three alien introgression lines. The results and resources developed from this study will support further analysis of the genomic structure of 6VS and cloning of potential functional RGA genes and accelerate its utilization in breeding for bread wheat's improvement.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11091695/s1. Figure S1: Sequence length distribution of 3275 coding genes (A) and their Gene Ontology classification (B). Figure S2: Microsynteny between two scaffolds scaffold6533 (A) and scaffold16282 (B) and their respective collinear counterparts in barley. Figure S3: The amplification results of representative markers in four *Haynaldia villosa* lines and three *H. villosa* 6VS additional lines. Table S1: Markers specific for short arm of *H. villosa* 6V chromosome. Table S2: The amplification results of all IT markers in multiple *Haynaldia villosa* lines.

Author Contributions: X.W. and J.D. conceived, designed, and coordinated the work; J.V. and J.D. flow-sorted telosome 6VS, determined the purity in flow-sorted fractions and amplified chromosomal DNA; K.H. sequenced amplified chromosomal DNA; X.Z., W.W., J.D. and H.W. wrote the manuscript; X.Z., W.W., M.L., Z.Y. and J.L. performed experiments; J.X., W.W., X.Z. and Y.W. analyzed the data. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Key Research and Development Program (2016YFD0101004, 2016YFD0102001-004), the National Natural Science Foundation of China (Nos. 31571653, 31771782 and 31201204), the International Cooperation and Exchange of the National Natural Science Foundation of China (No. 31661143005), the special fund of Jiangsu Province for the transformation of scientific and technological achievements (BA2017138), the Creation of Major New Agricultural Varieties in Jiangsu Province (PZCZ201706), the SAAS Program for Excellent Research Team, the Science and Technology Service Programs of the Chinese Academy of Sciences (KFJ-STS-ZDTP-002), the Jiangsu Agricultural Technology System (JATS) (No. 2019429), the Key Research and Development Major Project of Ningxia Hui Autonomous Region (No. 2019BBF02022-04), and the Plants as a tool for sustainable global development (No. CZ.02.1.01/0.0/0.0/16_019/0000827).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequence read data of 6VS chromosome was deposited in the (NCBI) Sequence Read Archive (SRA) and is available under accession number SRR10492095.

Acknowledgments: We also thank Zdeňka Dubská, Marie Kubaláková, Romana Šperková and Jitka Weiserová for the assistance with chromosome sorting and DNA amplification.

Conflicts of Interest: The authors declare no conflict of interest.

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