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ORIGINAL ARTICLE

Chromosomal mapping of a major genetic locus from *Agropyron cristatum* chromosome 6P that influences grain number and spikelet number in wheat

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Abstract

Key message **A novel locus on *Agropyron cristatum* chromosome 6P that increases grain number and spikelet number was identified in wheat–*A. cristatum* derivatives and across 3 years.**

Abstract *Agropyron cristatum* ($2n = 4x = 28$, PPPP), which has the characteristics of high yield with multiple flowers and spikelets, is a promising gene donor for wheat high-yield improvement. Identifying the genetic loci and genes that regulate yield could elucidate the genetic variations in yield-related traits and provide novel gene sources and insights for high-yield wheat breeding. In this study, cytological analysis and molecular marker analysis revealed that del10a and del31a were wheat–*A. cristatum* chromosome 6P deletion lines. Notably, del10a carried a segment of the full 6PS and 6PL bin (1–13), while del31a carried a segment of the full 6PS and 6PL bin (1–8). The agronomic characterization and genetic population analysis confirmed that the 6PL bin (9–13) brought about an increase in grain number per spike (average increase of 10.43 grains) and spikelet number per spike (average increase of 3.67) over the three growing seasons. Furthermore, through resequencing, a multiple grain number locus was mapped to the physical interval of 593.03–713.89 Mb on chromosome 6P of *A. cristatum* Z559. The RNA-seq analysis revealed the expression of 537 genes in the del10a young spike tissue, with the annotation indicating that 16 of these genes were associated with grain number and spikelet number. Finally, a total of ten *A. cristatum*-specific molecular markers were developed for this interval. In summary, this study presents novel genetic material that is useful for high-yield wheat breeding initiatives to meet the challenge of global food security through enhanced agricultural production.

Abbreviations

GNS	Grain number per spike
SNS	Spikelet number per spike
FFNS	Fertile floret number per spikelet
TGW	Thousand-grain weight
GISH	Genomic in situ hybridization
FISH	Fluorescence in situ hybridization

Yida Lin and Shenghui Zhou have contributed equally to this work.

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Introduction

Common wheat (*Triticum aestivum*, $2n = 6x = 42$, AABBDD) is a major crop that feeds approximately 40% of the world's population and provides 20% of all human calories and protein (Calderini et al. 2020; Xiao et al. 2022). Increasing grain number per spike (GNS) is an important way to increase grain yield while facing the challenges of increasing population and shrinking farmland area (Duan et al. 2018; Miransari and Smith 2019). GNS can be physically and genetically divided into two subcomponents: the spikelet number per spike (Boden et al. 2015; Glenn et al. 2023; Li et al. 2021) and the fertile

floret number per spike (Sakuma et al. 2019; Zhang et al. 2022). However, there have been limitations to improvements in wheat, as there is a lack of novel genes and germplasms exhibiting high-yield potential.

The transfer of excellent genetic variation from wheat relatives to common wheat through distant hybridization is an effective method for enriching the genetic diversity of wheat (Li et al. 2008). For example, the wheat–rye ($2n = 2x = 14$, RR) T1RS·1BL translocation line is widely used in high-yield and disease-resistant wheat breeding (Hackauf et al. 2022; Mago et al. 2005; Mater et al. 2004), and approximately 1050 wheat cultivars that carry the T1RS·1BL translocation inherit the chromosome arm 1RS of the rye cultivar Petkus (Schneider et al. 2016). In addition, the powdery mildew resistance gene *Pm21* from *Dasypyrum villosum* ($2n = 2x = 14$, VV), which is widely used in wheat disease resistance breeding in China, was cloned by map-based cloning and next-generation sequencing strategies (He et al. 2018; Xing et al. 2018). Therefore, identification of the ideal alien donor and introduction of high-yield genes into wheat are feasible for high-yield breeding.

Agropyron cristatum ($2n = 4x = 28$, PPPP) is an alien donor involved in wheat improvement. It has a great number of spikelets and flowers, and has the ability to confer resistance to various biotic and abiotic stresses (Farashi and Karimian 2021; Jiang et al. 2018; Li et al. 2016; Lin et al. 2023). In recent studies, we demonstrated that chromosome 6P was responsible for significant increases in the GNS, spikelet number per spike (SNS) and fertile floret number per spikelet (FFNS) (Wu et al. 2006), and the transfer of chromosome 6P into wheat has potential application value for increasing wheat yield. To narrow the physical interval of genes that regulate the grain number and to develop novel germplasms for wheat breeding, many translocation and deletion lines have been created by radiation with different segments from the chromosome 6P addition line 4844-12 (Song et al. 2013). In recent years, we confirmed that the genetic effect on increasing grain number was derived from chromosome 6PL (Zhang et al. 2019).

To further pinpoint the genetic loci regulating grain number and explore potential candidate genes, two novel deletion lines del10a (6PS·6PL bin 1–13) and del31a (6PS·6PL bin 1–8) were developed in this study. Through cytological, molecular marker and genetic analyses of agronomic traits, the enhanced-GNS locus was mapped to the 6PL bin (9–13). In this interval, the annotations of 16 genes were associated with grain number and were expressed in young spike of del10a. Additionally, we have developed ten specific molecular markers for *A. cristatum* that can be utilized in marker-assisted selection for this multi-grain genetic locus. In conclusion, we found a novel locus for increasing grain number and spikelet number in the 6PL bin (9–13), laying a foundation for further mapping, cloning and using *A. cristatum* genes for wheat breeding.

Materials and methods

Plant materials

The common wheat–*A. cristatum* (Z559, PPPP, $2n = 4x = 28$; from Xinjiang) deletion lines del10c (6PL bin 1–13) (Song et al. 2016a), del31a (6PS·6PL bin 1–8) and del10a (6PS·6PL bin 1–13) were derived from the chromosome 6P disomic addition line 4844-12 through radiation induction. Specifically, del10c (6PL bin 1–13) and del31a (6PS·6PL bin 1–8) were generated via five consecutive backcrosses with Fukuhokomugi (Fukuho; AABBDD, $2n = 6x = 42$; sourced from Japan). In parallel, del10a was developed through five consecutive backcrosses with Gaocheng8901 (AABBDD, $2n = 6x = 42$; from China). The BC_5F_2 and BC_6F_2 populations of del10c were subjected to genetic analysis, and the positive plants characterized by the presence of *A. cristatum* chromatin and negative plants lacking *A. cristatum* chromatin within the populations were distinguished by *A. cristatum*-specific repeat sequence markers, namely *Acpr3a* and *Acpr7*, which can be used to detect the chromatin of *A. cristatum* in wheat (Han et al. 2016). Del19b and del21 are also deletion lines; del19b has only chromosome 6PL, and del21 has only chromosome 6PS (Zhang et al. 2019). The details of all the materials are presented in Table S1. All materials were maintained at Prof. Lihui Li's laboratory at the Institute of Crop Science, Chinese Academy of Agricultural Sciences.

GISH and FISH analysis

Genomic in situ hybridization (GISH) was performed to detect the chromosomes of *A. cristatum* (Z559) in del10a and del31a. The chromosomes of the root tip cells were analyzed according to the methods of Chen and Armstrong (1994). Z559 genomic DNA (gDNA) was labeled with Texas-Red-5-dCTP (red) as a GISH probe, while Fukuho gDNA was used as a block, and the ratio of probe to block was 1:40. The root tip cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence in situ hybridization (FISH) was performed with the oligonucleotide probes Oligo-pSc119.2-1 (green) and Oligo-pTa535-1 (red) to determine the integrity of the alien chromosomes and the variation in the wheat chromosomes (Tang et al. 2014). A similar procedure was performed according to the methods of Han et al. (2006, 2009), and all probes were synthesized by Sangon Biotech

(Shanghai). Fluorescent signals were observed via an Olympus AX80 fluorescence microscope (Olympus Corporation, Tokyo, Japan), imaged with a charge-coupled device (CCD) camera (Diagnostic Institute, Inc. Sterling Height, MI, USA) and modified with Photoshop CS6.

Chromosome 6P-specific molecular marker genotyping for del10a and del31a

Previously, Zhang et al. (2015) designed the chromosome 6P-specific expressed sequence tag-sequence tagged site (EST-STS) marker according to the transcriptome of *A. cristatum*, and Song et al. (2016b) used deletion and translocation lines to divide the chromosome 6P into 31 regions (6PS 14 regions, 6PL 17 regions) and mapped 255 6P-specific EST-STS markers to 31 regions. Among them, a total of thirty-one 6P-specific markers from 31 bins (one marker from each of the bins) were selected for detecting the segments of chromosome 6P in del10a and del31a. Details of the molecular markers used are shown in Table S2. All primers were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China).

A modified cetyltrimethyl ammonium bromide (CTAB) method was used to extract genomic DNA from all plant materials (Carra et al. 2007). A total of 20 µl of reaction mixture was used for PCR, which consisted of 10 µl of Green Master Mix (Nanjing Vazyme Biotech Co., Ltd.), 1.2 µl of DNA template (100 ng/µl), 1 µl of each primer (2 µM) and 6.8 µl of ddH_2O. Each PCR was performed as follows: one cycle at 94 °C for 5 min for denaturation; 35 cycles of 94 °C for 30 s, 56–58 °C (depending on the annealing temperature for each marker) for 30 s and 72 °C for 30 s for extension; one cycle at 72 °C for 10 min for final extension; and maintenance at 4 °C. The PCR products were assessed by 1% agarose gel electrophoresis.

Analysis of agronomic characteristics

All materials were planted at the Xinxiang Experimental Base of Henan, Institute of Crop Science, Chinese Academy of Agricultural Sciences, during three growing seasons in 2020–2021, 2021–2022 and 2022–2023. Each row was 2 m long and was planted with 20 seeds; and the seeds were approximately 10 cm apart, and the row spacing was 25 cm. The deletion lines del10a, del10c and del31a and the recurrent parent Fukuho and Gaocheng8901 were randomly planted in the field over three growing seasons. Additionally, both positive and negative plants in the BC_5F_2 and BC_6F_2 genetic populations of del10c were also randomly planted. The plant height (PH, cm), spike length (SL, cm), effective tillering (ET), SNS, FFNS, GNS and thousand-grain weight (TGW, g) were determined after harvest. The measurement methods for each trait were as follows: PH is the height of the plant, calculated from the base of the stem to the top spikelet, not including the root length; SL indicates the length of the main spike, measured by the distance from the base spikelet to the top spikelet, not including the awn length; SNS represents the total number of spikelets in the main spike, including sterile spikelets and fertile spikelets; ET is the number of spikes per plant; FFNS is the largest kernel number in a spikelet from the middle region; GNS is the total grain number in the main spikelet; and TGW was measured by an SC-G seed detector (Hangzhou WSeen Detection Technology Co., Ltd.). The significant differences in the agronomic characteristics of Fukuho, Gaocheng8901, del10a, del31a and del10c were determined using one-way ANOVA at a probability level of $p = 0.05$. The significant differences in positive and negative plants of the del10c genetic populations were determined using Student's t test at the probability levels of $p < 0.05$ (*) and $p < 0.01$ (**).

Resequencing analysis and molecular marker development

The leaves of del10a and del31a were collected at the seedling stage for resequencing to determine the physical interval of the 6PL bin (9–13) at Huazhi Biotech Co., Ltd. (Changsha, China). The DNA libraries were generated using MGIEasy DNA library preparation kits following the manufacturer's recommendations, and the libraries were sequenced using the BGISEQ-500 platform with a paired-end read length of 150 bp (Lin et al. 2022). The sequencing depth was approximately 1X. The raw data were filtered by "fastp" tools (Chen et al. 2018), and the clean reads were mapped to the integrated *A. cristatum* Z559 reference sequences (unpublished) and the Chinese Spring (CS) RefSeqv1.0 (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v1.0/) using the BWA tool (Houtgast et al. 2018). BEDTools was used to construct windows for the genome and calculate the sequencing coverage of reads in each window, for which the window size was 1 Mb (Quinlan and Hall 2010). The ggplot2 package of R was used to visualize read coverage (Gustavsson et al. 2022). The genes associated with grain number and spikelet number were obtained via trait annotation and domain annotation. SAMtools was used to extract gene sequences (Li et al. 2009), the BLASTN tool was used to identify SNP differences between *A. cristatum* and wheat CS (Ye et al. 2012), and primers were designed for SNP loci and synthesized by Shanghai Sangon Biotech Co., Ltd. PCR was the same as that used for the EST-STS markers. The bands present in 4844-12 and del10a but absent in Fukuho, Gaocheng8901 and del31a were identified as specific markers.

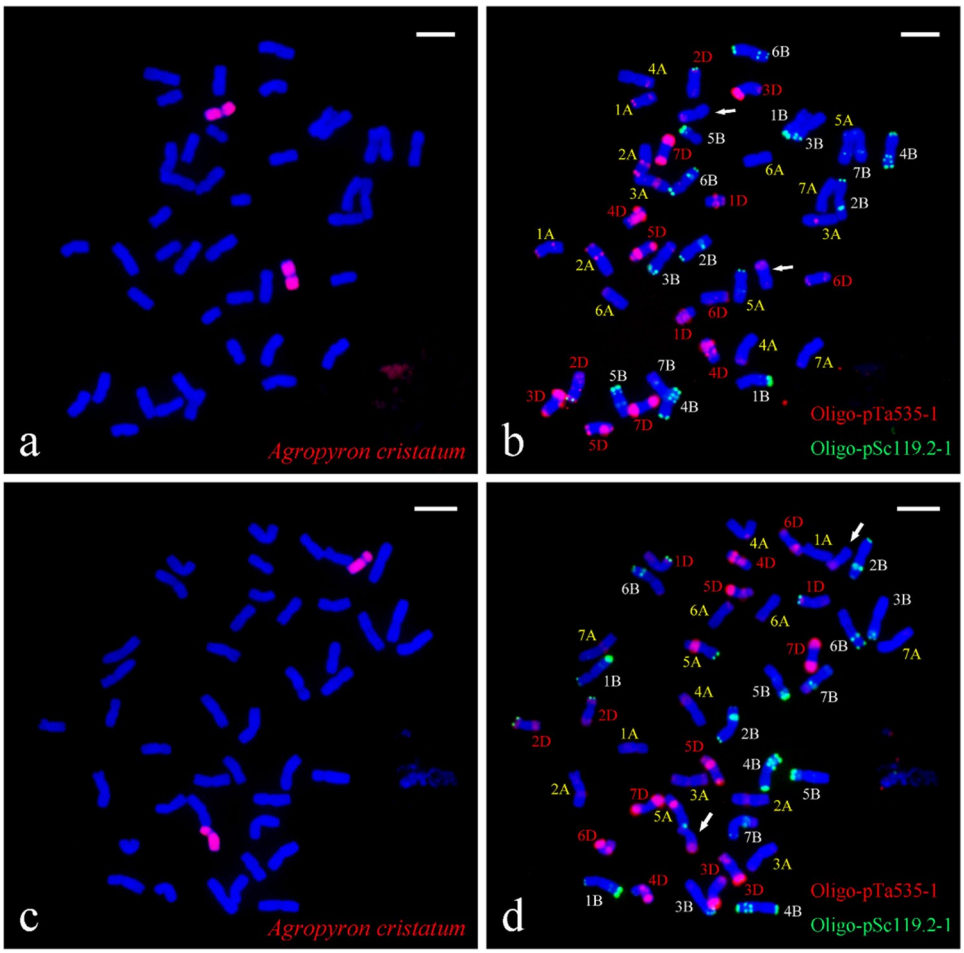


Fig. 1 Cytogenetic analysis of del10a and del31a by in situ hybridization. GISH (**a**) and FISH (**b**) analysis of del10a; GISH (**c**) and FISH (**d**) analysis of del31a. Chromosomes were counterstained with DAPI and visualized with blue fluorescence. The red signal in **a** and **c** represents the chromosome of *Agropyron cristatum*, the FISH probe Oligo-pTa535-1 is shown in red, and Oligo-pSc119.2-1 is shown in green in **b** and **d**. Scale bar = 10 μm

RNA-seq analysis

RNA was extracted from the young spike of del10a at the double ridge stage using TRIzol reagent (Thermo Fisher Scientific Inc., Shanghai, China) according to the manufacturer's instructions. The RNA libraries were sequenced on the Illumina sequencing platform by Smartgenomics Technology Institute (Tianjin, China). One biological replicate was used for young spike tissue, and the sequencing volume was 10 G for each sample. The clean data were obtained by removing the reads containing adaptors, poly-N sequences and low-quality reads using the "fastp" tool (Chen et al. 2018). The clean data from each sample were separately aligned to the wheat CS reference genome (IWGSC RefSeq v1.0) and the reference genome of Z559 by the HISAT2 tool (Kim et al. 2019). Then the count of each gene was calculated by the featureCounts tool (Liao et al. 2014).

Real-time reverse transcription PCR (qRT-PCR)

The total RNA from the leaf, sheath, stem and young spike of del10a were extracted and reverse transcribed into cDNA for qRT–PCR using a Reverse Transcriptase Kit (ZOMANBIO, Beijing, China). The spike development genes from 6PL bin (9–13) were selected for qRT–PCR, and the qRT–PCR primers were designed using Primer 5 software. The qRT–PCR was performed using a TB Green®® Premix Ex Taq™ II (Takara, Osaka, Japan) kit in a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Three technical replicates were set for each biological sample, and the relative quantification of gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The wheat *Actin* (*TraesCS1B02G024500*) gene was used as the housekeeping gene to calibrate the expression levels of genes.

Results

Chromosome composition analysis of del10a and del31a

Tracking of the chromatin of *A. cristatum* in wheat was enabled with GISH by using gDNA from *A. cristatum* as a probe, and FISH was used to detect chromosome variation by using the oligonucleotide probes Oligo-pSc119.2-1 and Oligo-pTa535-1. The results revealed that both del10a

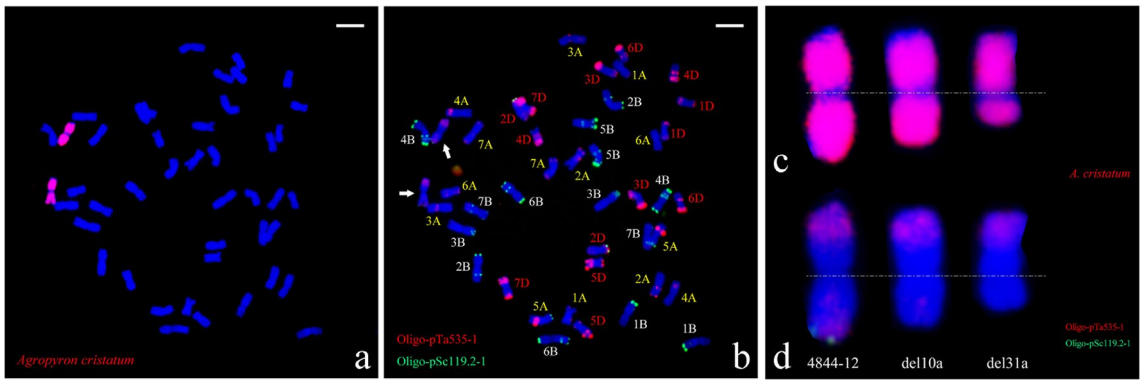


Fig. 2 Chromosome length analysis of del10a and del31a. GISH (**a**) and FISH (**b**) analysis of 4844-12; (**c**) and (**d**) comparison of fluorescence signals between del10a, del31a and 4844-12. The FISH probes Oligo-pTa535-1 (red) and Oligo-pSc119.2-1 (green) are shown in **b** and **d**, and the GISH probes (red) are shown in **a** and **c**. Scale bar = 10 μm (color figure online)

and del31a had forty-two wheat chromosomes with blue 4',6-diamidino-2-phenylindole (DAPI) signals and two 6P chromosomes from *A. cristatum* with red signals (Fig. 1), were used for genotyping. The results demonstrated that 14 markers from 6PS had specific bands amplified in 4844-12 and del21; 17 markers from 6PL had specific bands ampli-

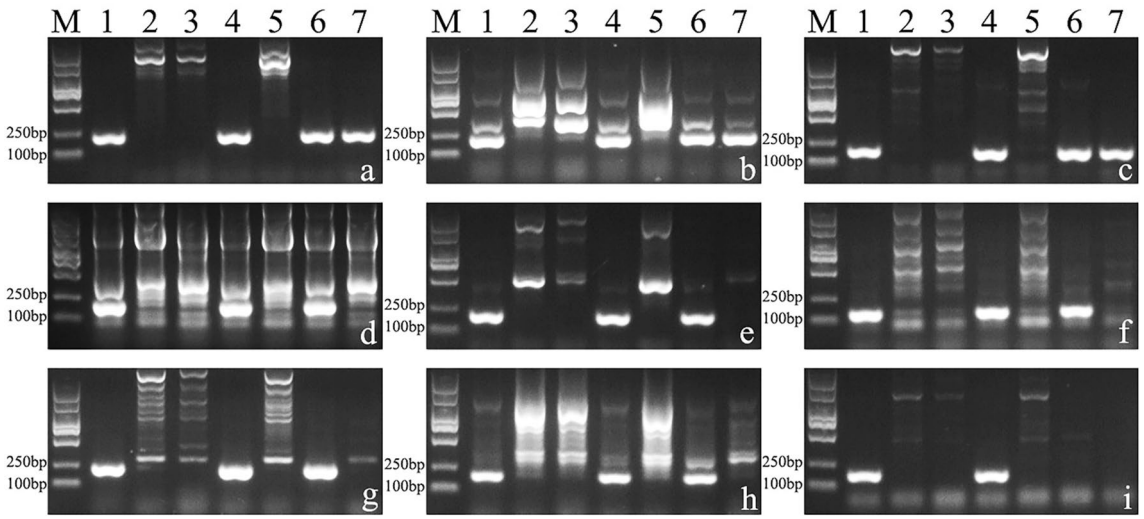


Fig. 3 6P-specific molecular marker genotyping for del10a and del31a. **a** *AgC5385*; **b** *AgC5103*; **c** *AgC15870*; **d** *AgC5532*; **e** *AgC2453*; **f** *AgC32015*; **g** *AgC9467*; **h** *AgC3960*; **i** *AgC10192*. M: D2000 puls; 1: 4844-12; 2: Fukuho; 3: Gaocheng8901; 4: del19b; 5: del21; 6: del10a; 7: del31a

and compared with the chromosome 6P in the addition line 4844-12, the chromosome 6P in del10a and del31a was incomplete (Fig. 2a–c). The FISH analysis indicated that chromosome 6P in del10a and del31a lacked segments of the chromosome 6PL terminus, and the segment of chromosome 6P in del10a was larger than that in del31a (Fig. 2d). Therefore, del10a and del31a are wheat–*A. cristatum* chromosome 6P deletion lines with different segments.

To further determine the segments in del10a and del31a, a total of thirty-one 6P-specific EST-STS molecular markers fied in 4844-12 and del19b; all 31 markers were absent in the recurrent parents Fukuho and Gaocheng8901; four markers (*Agc10192*, *Agc33878*, *Agc26468* and *Agc8139*) from the 6PL bin (14–17) were missing the specific bands in del10a and del31a; and five markers (*Agc5532*, *Agc2453*, *Agc32015*, *Agc9467* and *Agc3960*) from the 6PL bin (9–13) were also missing the specific bands in del31a (Table S3, Fig. 3). Therefore, we confirmed that del10a had a segment with the full 6PS and 6PL bin (1–13), while del31a carried a segment with the full 6PS and 6PL bin (1–8).

Table 1 Analysis of agronomic traits during three growing seasons

Years	Materials	Generations	Plant number	Plant height (cm)	Spike length (cm)	Effective tillering	Spikelet number per spike	Fertile floret number per spikelet	Grain number per spike	Thousand-grain weight (g)
2020–2021	Fukuho		10	103.60 ± 2.63b	11.78 ± 0.67a	19.90 ± 3.98a	20.50 ± 0.53c	4.20 ± 0.42b	61.00 ± 4.83b	37.35 ± 1.78a
	del31a	M_3	10	99.40 ± 6.96bc	10.55 ± 1.01b	12.70 ± 3.71b	20.20 ± 1.55c	3.90 ± 0.32b	61.10 ± 5.67b	34.49 ± 2.91a
	Gaocheng8901		10	88.40 ± 2.80c	9.74 ± 0.41b	9.30 ± 1.06b	23.10 ± 0.88b	4.40 ± 0.52ab	63.00 ± 5.72b	34.55 ± 5.11a
	del10a	M_3	10	115.60 ± 9.38a	8.69 ± 1.02c	12.30 ± 1.70b	26.60 ± 0.97a	4.90 ± 0.57a	74.00 ± 8.27a	38.28 ± 4.13a
2021–2022	Fukuho		10	101.10 ± 4.56b	11.49 ± 0.83a	16.10 ± 3.41a	20.50 ± 0.71c	4.00 ± 0.47b	60.80 ± 6.09bc	31.65 ± 2.33c
	del31a	M_4	10	93.50 ± 6.42c	10.08 ± 0.48bc	11.50 ± 3.75b	18.30 ± 0.48d	4.00 ± 0.00b	51.70 ± 4.45c	32.35 ± 2.04bc
	Gaocheng8901		10	85.65 ± 3.42d	9.55 ± 0.50c	12.20 ± 2.15b	22.90 ± 1.10b	4.40 ± 0.52b	61.80 ± 9.73b	34.24 ± 1.81b
	del10a	M_4	10	107.40 ± 4.17a	8.96 ± 0.45c	12.40 ± 2.46ab	26.60 ± 1.07a	4.80 ± 0.42ab	72.70 ± 5.21a	38.19 ± 1.79a
	del10c	M_4	10	110.30 ± 4.85a	10.40 ± 0.77b	11.00 ± 3.06b	22.20 ± 1.14b	5.00 ± 0.47a	81.60 ± 9.52a	31.91 ± 1.01bc
	del10c/6P+	BC_5F_2	67	111.34 ± 4.17	12.42 ± 0.61	12.79 ± 3.15	20.73 ± 0.83**	5.01 ± 0.54**	72.60 ± 7.61**	–
	del10c/6P-	BC_5F_2	120	110.38 ± 5.16	12.98 ± 10.06	13.63 ± 3.55	20.09 ± 0.82	4.52 ± 0.53	64.11 ± 7.58	–
2022–2023	Fukuho		10	105.20 ± 3.61b	11.46 ± 0.96b	15.20 ± 3.61a	20.60 ± 0.84c	4.10 ± 0.32b	57.10 ± 6.52c	32.63 ± 1.33b
	del31a	M_5	10	98.50 ± 6.00b	10.18 ± 0.45c	12.30 ± 3.20a	18.40 ± 0.84d	4.20 ± 0.42b	55.20 ± 6.05c	31.81 ± 3.78b
	Gaocheng8901		10	89.30 ± 1.34c	10.08 ± 0.47c	12.20 ± 3.16a	23.00 ± 0.82b	4.30 ± 0.48b	65.50 ± 6.62b	33.69 ± 3.24ab
	del10a	M_5	10	111.80 ± 9.13ab	8.61 ± 0.76d	13.90 ± 5.63a	26.80 ± 1.03a	4.60 ± 0.70ab	74.90 ± 8.61a	36.24 ± 1.87a
	del10c	M_5	10	114.30 ± 4.45a	12.71 ± 0.38a	11.90 ± 3.14a	21.60 ± 0.52c	5.00 ± 0.00a	74.40 ± 3.37a	32.14 ± 2.60b
	del10c/6P+	BC_6F_2	39	114.33 ± 4.82	11.33 ± 0.76**	10.82 ± 2.55	21.59 ± 0.97**	4.69 ± 0.61**	67.64 ± 7.49**	–
	del10c/6P-	BC_6F_2	89	112.82 ± 4.63	10.89 ± 0.66	13.28 ± 3.92**	20.93 ± 1.04	4.28 ± 0.56	61.44 ± 7.30	–

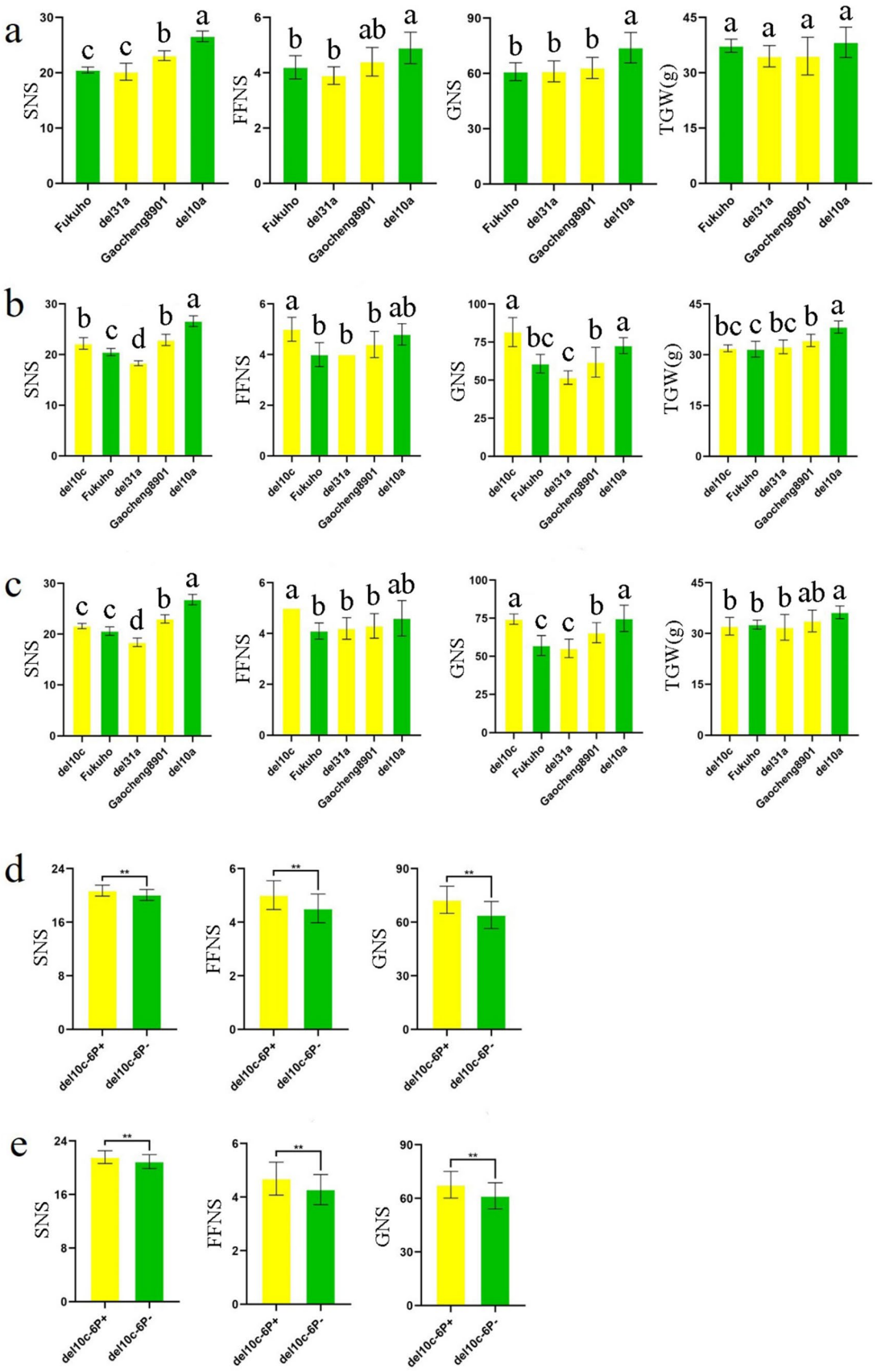
The significant differences in the agronomic characteristics of Fukuho, Gaocheng8901, del10a, del31a and del10c were determined using one-way ANOVA at a probability level of $p = 0.05$. The significant differences in positive and negative plants of the del10c genetic populations were determined using Student's t test at the probability levels of $p < 0.05$ (*) and $p < 0.01$ (**). The designation "6P+" indicates plants carrying *A. cristatum* 6P chromatin, while "6P−" indicates plants lacking *A. cristatum* 6P chromatin

Fig. 4 Agronomic traits of del10a, Gaocheng8901, del31a and Fukuho in the field. 1: del10a; 2: Gaocheng8901; 3: del31a; 4: Fukuho. **a** and **f** Plants; **b** and **g** Front view of the spike; **c** and **h** Side view of a stalk of the spike; **d**, **e**, **i** and **j** Spikelet. The spikes were chosen from the primary stem, while the spikelets were specifically selected from the central region of the spike. Scale bar in **a** and **f** = 10 cm; scale bar in **b**, **c**, **g** and **h** = 1 cm; scale bar in **d**, **e**, **i** and **j** = 0.5 cm

Evaluation of agronomic traits and location of multiple grain number locus

The agronomic performance of del10a, del31a, del10c (6PL bin 1–13), Fukuho and Gaocheng8901 were investigated during the three growing seasons of 2020–2021, 2021–2022 and 2022–2023 in Xinxiang, Henan Province. The results indicated that, compared with the GNS, SNS and PH of Gaocheng8901, those of del10a increased significantly by 11.00, 10.90 and 9.40; by 3.50, 3.70 and 3.80; and by 27.20 cm, 21.75 cm and 22.50 cm, respectively, during the three growing seasons (p value < 0.01); and compared with the spike length of Gaocheng8901 (recurrent parent), that of del10a decreased significantly by 1.05, 0.59 and 1.47 cm, respectively (p value < 0.05). Compared with the TGW of Gaocheng8901, that of del10a increased significantly by 3.95 g and 2.55 g in the 2021–2022 and 2022–2023 growing seasons, respectively, while FFNS and ET showed no significant differences. In addition, compared with Fukuho (a recurrent parent), del31a did not exhibit significant increases in GNS, FFNS, TGW and SNS during the three growing seasons, and the del10c exhibited significant increases in GNS, FFNS and SNS (p value < 0.01); moreover, TGW was not significantly different between the 2021–2022 and 2022–2023 growing seasons. In addition, compared with del31a, del10a exhibited significant increases in GNS, FFNS and SNS by 12.90, 21.00 and 19.70; by 1.00, 0.80 and 0.40; and by 6.40, 8.30 and 8.40, respectively, in the three years (p value < 0.05) (Table 1, Figs. 4, 5a–c). Therefore, we speculate that the 6PL bin (9–13) in del10a has genetic benefits that steadily increase GNS and SNS (Fig. 6a).

To further confirm the genetic effects of the 6PL bin (9–13) in the Fukuho background, the agronomic traits of the BC_5F_2 and BC_6F_2 genetic populations of the deletion line del10c (carrying 6PL bin 1–13) were analyzed during the 2021–2022 and 2022–2023 growing seasons. A total of 67 positive plants and 120 negative plants in the BC_5F_2 population and 39 positive plants and 89 negative plants in the BC_6F_2 population were identified by the *A. cristatum* repeat sequence markers *Acpr3a* and *Acpr7*. Compared with the SNS, FFNS and GNS in the negative plants, those in the BC_5F_2 population significantly increased by 0.64, 0.49 and 8.49, representing increases of 3.19%, 10.84% and 13.24%, respectively (p value < 0.01). Similarly, compared with those


a
SNS
FFNS
GNS
TGW(g)
Fukuho
del31a
Gaocheng8901
del10a
b
del10c
c
d
e
del10c-6P+
del10c-6P-

◂**Fig. 5** Significance analysis of agronomic traits. **a** Significance analysis of Fukuho, del31a, Gaocheng8901 and del10a regarding SNS, FFNS, GNS and TGW in 2020–2021; **b** Significance analysis of Fukuho, del10c, del31a, Gaocheng8901 and del10a regarding SNS, FFNS, GNS and TGW in 2021–2022; **c** Significance analysis of Fukuho, del10c, del31a, Gaocheng8901 and del10a regarding SNS, FFNS, GNS and TGW in 2022–2023; **d** Significance analysis of BC_5F_2 population of del10c regarding SNS, FFNS and GNS in 2021–2022; **e** Significance analysis of BC_6F_2 population of del10c regarding SNS, FFNS and GNS in 2022–2023

in the negative plants, the SNS, FFNS and GNS in the BC_6F_2 population significantly increased by 0.66, 0.41 and 6.20, respectively, representing increases of 3.15%, 9.58% and 10.09%, respectively (p value < 0.01) (Table 1, Fig. 5d, e). Therefore, these results indicated that the 6PL bin (9–13) is a novel locus that is mainly associated with enhanced GNS and SNS.

Physical mapping of the multiple GNS locus

To determine the accuracy of the physical interval of the 6PL bin (9–13), del10a and del31a were resequenced and mapped to the *A. cristatum* Z559 reference genome by the BWA tool. By calculating the chromosome coverage of the reads, we determined that del10a carried an approximately 0–713.89 Mb interval of chromosome 6P, while del31a carried an approximately 0–593.03 Mb interval of chromosome 6P. Therefore, the multiple grain number locus was located within the 593.03–713.89 Mb region of the 6P chromosome (Fig. 6b). According to the results of de novo prediction, homology searches and transcript-based assembly, a total of 993 protein-coding genes (*Ac6P02G357100–Ac6P02G456300*) were deposited in the locus of the 6PL bin (9–13).

RNA-seq for del10a and tissue-specific expression analysis for spike development genes

To further investigate the tissue expression of the 993 genes in the 6PL bin (9–13), the young spike of del10a were collected at the double ridge stage for RNA-seq. A total of 537 genes were expressed in young spike. Among these genes, sixteen were associated with spikelet number, spikelet fertility and filled grain number, including five AP2 genes and one MADS-box gene (Table S4). To further characterize these genes, their cDNA sequences were extracted from *A. cristatum* genome sequences and subsequently utilized for the development of *A. cristatum*-specific cDNA markers to facilitate tissue-specific expression analysis. Four genes, namely *Ac6P02G384400*, *Ac6P02G393000*, *Ac6P02G398000* and *Ac6P02G410600*, successfully generated their specific cDNA markers, denoted as *Ac-cDNA3844-1*, *Ac-cDNA3930-1*, *Ac-cDNA3980-1 and Ac-cDNA4106-2*, respectively (Table S5, Fig. 7a). The result of qRT–PCR demonstrated that *Ac6P02G384400*, *Ac6P02G393000* and *Ac6P02G410600* exhibited expression in young spike, stem and sheath tissues, while their expression levels were extremely low in leaf tissue. And *Ac6P02G398000* displayed high expression levels across all four tissues (Fig. 7b). However, further work is needed to verify whether these genes indeed function in young spike development of wheat.

Development of specific markers at the locus of multiple GNS

To efficiently trace the chromatin of the 6PL bin (9–13) in wheat, the sequences of ten genes from this interval were extracted from Z559 via SAMtools, the different SNP loci were determined via the BLASTN tool and compared against the wheat CS reference genome, and a total of ten dominant molecular markers were obtained (Table S6). PCR and agarose gel electrophoresis revealed that all the markers with specific bands were amplified from 4844-12 and del10a, while Fukuho, Gaocheng8901 and del31a had none (Fig. 8). In the future, these markers may enable accurate tracing of *A. cristatum* chromatin in wheat and provide a marker tool for subsequent mining and cloning of high-yield genes to increase grain number and spikelet number.

Discussion

A. cristatum provides germplasm and gene resources for high-yield wheat breeding

Creating new germplasms through distant hybridization with distant relatives and making those germplasms available to breeders are important methods for addressing the issue of the narrowing gene pools in wheat breeding. For example, in terms of providing germplasm and genetic resources for wheat resistance breeding, wheat–rye derivative lines from chromosomes 1R, 4R and 6R with powdery mildew resistance (Han et al. 2020; Ma et al. 2020; Perovic et al. 2015); wheat–*Thinopyrum ponticum* translocation line WTT80 with powdery mildew resistance; WTT34 with stem rust resistance (Yang et al. 2021a, 2022); wheat–*Thinopyrum elongatum* translocation lines Zhongke 1878, Zhongke 166 and Zhongke 545 with Fusarium head blight resistance (Guo et al. 2023); and wheat–*Thinopyrum intermedium* translocation line WTT11 with stripe rust resistance have been developed in wheat breeding (Yang et al. 2021b).

However, there are few reports about the specific genetic traits present in wheat relatives that contribute to an increase in the number of grains and spikelets.

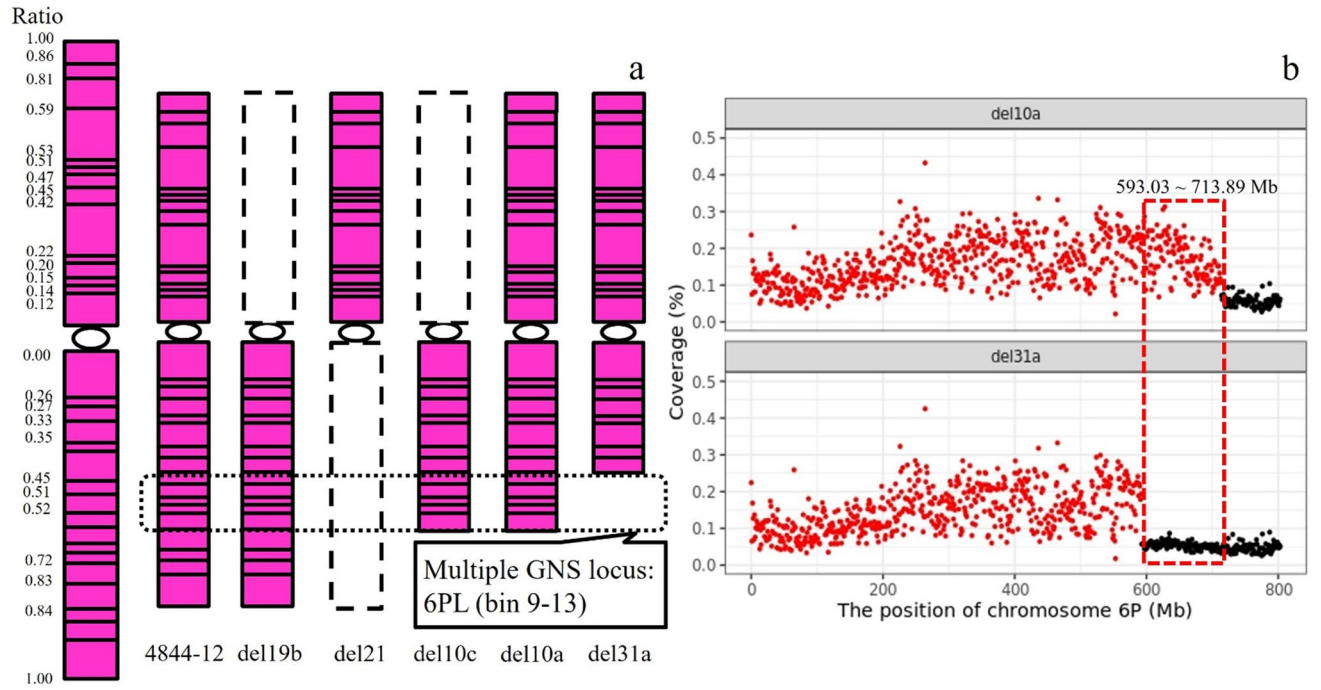


Fig. 6 Multiple GNS locus in the 6PL bin (9–13). **a** On the left are 31 chromosome bins of chromosome 6P. Pink represents *A. cristatum* chromatin. **b** Physical interval analysis of the 6PL bin (9–13) by resequencing. The horizontal coordinate represents the position of the 6P chromosome, and the ordinate represents read coverage at the corresponding location. The red dots represent the chromosome 6P of del10a and del31a, and the black dots represent missing parts of the chromosome

Fortunately, *A. cristatum*, which has multiple flowers and spikelets, is an ideal gene donor for high-yield wheat breeding. Li et al. (1998) achieved hybridization between common wheat and the distant relative *A. cristatum* in the 1990s, and the wheat–*A. cristatum* chromosome 6P addition line 4844-12, which exhibited significantly increased grain number, was developed (Wu et al. 2006). To map the genes that regulate grain number on chromosome 6P, many wheat–*A. cristatum* 6P translocation and deletion lines with different segments were created by radiation induction 4844-12 (Song et al. 2013). In this study, we produced two deletion lines, del10a and del10c, with high GNS and high SNS values and located a novel physical interval in the 6PL bin (9–13) that regulates grain number. These achievements have the potential to be used in wheat breeding to transfer the segment 6PL bin (9–13) into the wheat background in the form of small segment translocation lines by radiation induction. In summary, the deletion lines del10a and del10c presented in this study provide new germplasm and genetic resources for improving wheat yield.

The advantages and disadvantages of chromosomal mapping

Many deletion lines and translocation lines harboring different chromosome segments can be created by radiation induction, which can narrow the physical range of excellent genes. For example, the leaf rust resistance gene in the 2PL bin (0.66–0.86) (Jiang et al. 2018) and the adult powdery mildew resistance gene were found to be located in the 6PL bin (3–7) by deletion and translocation lines mapping (Lin et al. 2022). In addition, the blue-grained gene and powdery mildew resistance gene from *Thinopyrum ponticum* were located in bin 4AgL-6 with FL 0.75–0.89 and in the 3.79–97.12 Mb region of the 4AgS according to a similar method (Liu et al. 2018; Yang et al. 2023). Therefore, deletion and translocation lines mapping can narrow the physical interval of candidate genes at the chromosomal level to a certain extent. However, due to the segment size of the translocation and deletion lines, the target interval was too large to determine, and candidate genes could not be accurately screened. In this study, a total of 993 genes in the 120.86 Mb interval were identified by resequencing the deletion lines del10a and del31a, and a total of 16 potential candidate genes were annotated. These genes were expressed in the young spike at the double ridge stage. However, we cannot yet determine which genes regulate the GNS.

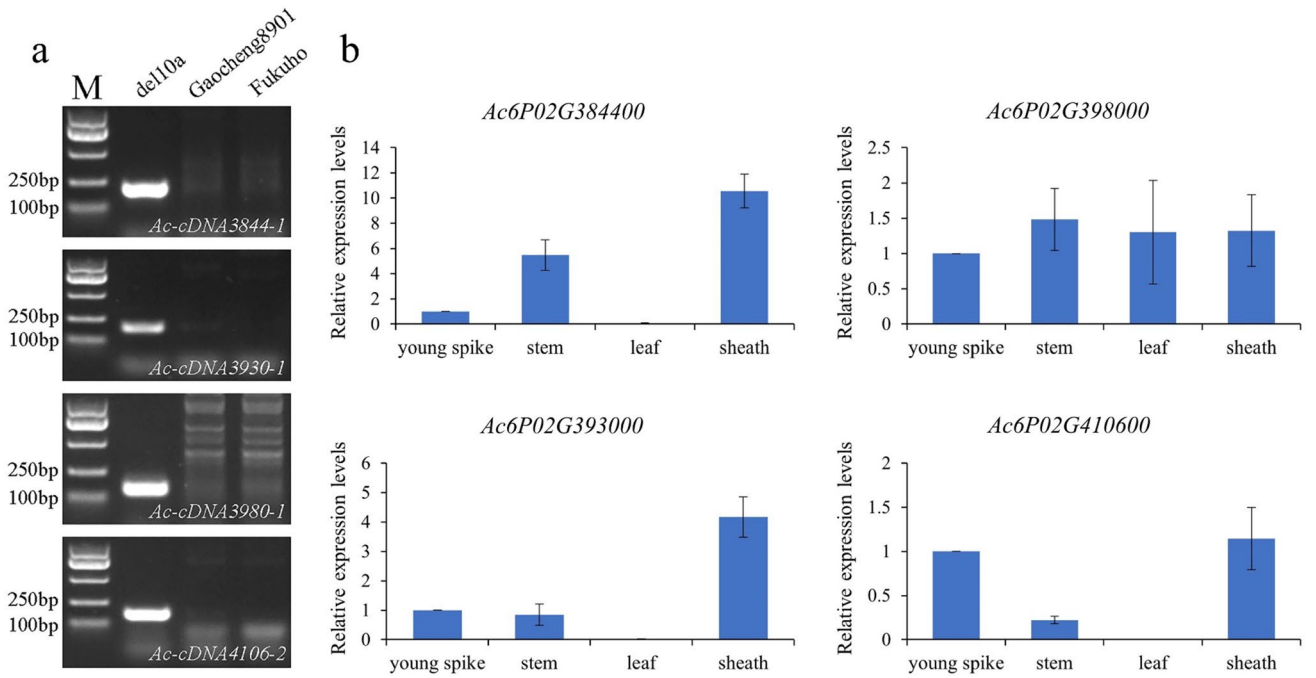


Fig. 7 *A. cristatum*-specific cDNA molecular marker development and tissue-specific expression analysis. **a** Development of *A. cristatum*-specific cDNA molecular marker for *A. cristatum* genes from 6PL bin (9–13); **b** The tissue-specific expression analysis of genes *Ac6P02G384400*, *Ac6P02G393000*, *Ac6P02G398000* and *Ac6P02G410600*

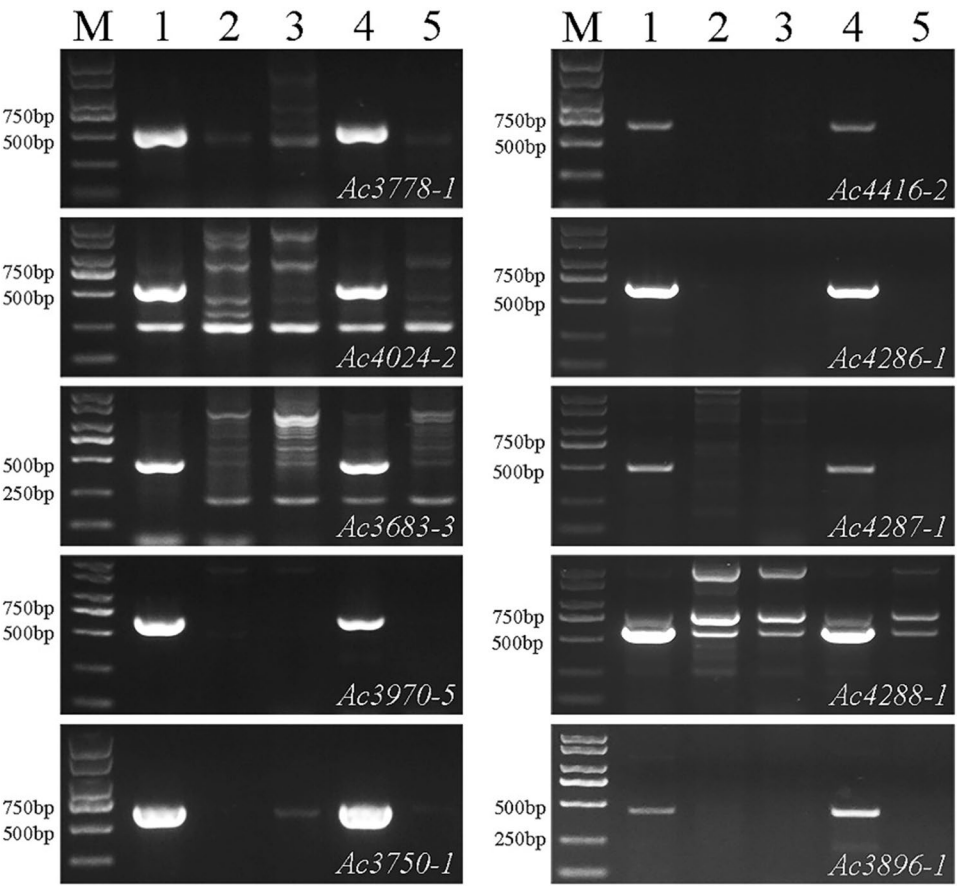


Fig. 8 Development of 6P-specific markers. M: D2000 plus. 1: 4844-12; 2: Fukuho 3: Gaocheng8901 4: del10a; 5: del31a

Therefore, in our future research, we plan to perform radiation induction on del10c, create translocation lines and deletion lines with smaller segments and backcross them with wheat cultivars to create new germplasms for high-yield wheat production and promote the cloning of multiple GNS genes.

The development of molecular markers facilitates the tracing of *A. cristatum* chromatin and cloning of candidate genes

In hybridization with distant relatives, much alien chromatin can be introduced into the wheat background to produce addition lines, translocation lines and introgression lines. The analysis of agronomic traits and GISH techniques can help identify alien chromatin in wheat. However, GISH does not detect small segments of alien chromatin at the chromosomal level. Therefore, the development of specific molecular markers is critical for tracing alien chromatin in wheat and applying this information to molecular marker-assisted breeding. In this study, we developed 10 PCR-based primers based on the SNP sites between *A. cristatum* and CS. For example, the markers *Ac3778-1* and *Ac4024-2* amplified specific bands in 4844-12 and del10a but not in Fukuho, Gaocheng8901 or del31a. Therefore, these markers can be used as convenient and efficient tools for detecting chromatin in the 6PL bin (9–13) of *A. cristatum* in wheat. On the other hand, these molecular markers provide a tool for further identifying multiple GNS genes in *A. cristatum* via map-based cloning in wheat.

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Author contribution statement LHL conceived the research. YDL performed the research. YDL and SHZ wrote the paper. WJY modified some pictures. BH, XZL and YXZ participated in part of the data collection and cytology work. JPZ, HMH, BJG, XMY, XQL and WHL participated in the preparation of the reagents and materials used in this study.

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Declarations

Conflict of interest The authors have not disclosed any competing interests.

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