# Introgression of Agropyron cristatum genes to counteract the negative relationship between grain number and grain weight of wheat

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**Research Article**

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## Abstract

*Agropyron cristatum* (2n = 4X = 28, PPPP), which harbour many high-yield and disease-resistance genes, is a promising donor for wheat improvement. Narrow genetic diversity and the trade-off between grain weight and grain number have become bottlenecks for increasing grain yield. In this study, WAT650I, a new translocation line was generated via radiation ($^{60}$Co-γ rays)-based mutation induction of chromosome 6P addition line 4844-12. Cytological analysis and molecular marker analysis revealed that WAT650I was a 6PL (bin ~ 12–17)·5BS-5BL translocation line. Assessment of agronomic traits and analysis of the $BC_4F_2$ and $BC_5F_2$ populations suggested that the 6PL terminal chromosome segment in WAT650I resulted in increased flag leaf length, plant height (PH), spikelet number per spike (SNS), kernel number per spikelet (KNS), grain number per spike (GNS) (average increased by 14.07 grains) and thousand-grain weight (TGW) (average increased by 4.31 g) during the growing seasons of 2020–2021 and 2021–2022. Additionally, the increased GNS locus and high-TGW locus of WAT650I were mapped to 6PL (16–17) and 6PL (12–13), respectively, by genetic population analysis of three translocation lines. In summary, by using high-yield genes of *A. cristatum* and overcoming the negative relationship between GNS and TGW associated with wheat breeding, we provide a valuable germplasm resource for broadening the genetic base of wheat.


## Key Message

Cytological, molecular markers and genetic population analysis indicated that the wheat 6PL (bin ~12-17)·5BS-5BL translocation line WAT650I presented a simultaneous increase in grain number per spike and thousand-grain weight.

## Introduction

Wheat is one of the most predominant crop species in the world and provides approximately 20% of the daily nutrients in human diets and livestock feed; therefore, a substantial increase in grain yield is needed to ensure food security (Foulkes et al. 2011; Shewry 2009). However, the narrow genetic diversity of wheat limits its genetic improvement (Reif et al. 2005). Increasing the grain number per spike (GNS), thousand-grain weight (TGW) and number of spikes per unit land area are three major methods to increase wheat yields (Wolde et al. 2019). However, the trade-off between grain weight and grain number has become a bottleneck for increasing grain yield, as demonstrated by recent studies of a wide range of wheat genotypes that included elite materials from the International Maize and Wheat Improvement Center (CIMMYT) (Calderini et al. 2021; Molero et al. 2019). Therefore, identifying valuable genetic resources that can simultaneously increase GNS and TGW of wheat is an important breeding goal.

Distant hybridization is an effective way to introduce excellent alien genes into wheat. For example, rye can be used to enhance disease resistance and yield; in particular, the *Lr26/Sr31/Yr9/Pm8* resistance gene clusters from the 1B·1R translocation line have been used in wheat production on a large scale (Mater et al. 2004; Yang et al. 2016). In addition, *Lr24/Sr24* resistance gene clusters and *Fhb7* from *Thinopyrum. ponticum* (Rai et al. 2021; Wang et al. 2020), *Pm21*, *Pm55*, *Pm62* from *Dasypyrum. villosum* (Gao et al. 2020; Zhang et al. 2016b; Zhang et al. 2018b) and *Lr19*, *Sr26*, *Sr43* form *Thinopyrum. elongatum* (Gennaro et al. 2009; Zhang et al. 2021; Niu et al. 2014) have been introgressed into the common wheat background, as these wheat relatives have provided an abundance genetic resources for breeding for increased resistance. Therefore, obtaining high-yield genes from alien relatives is a feasible approach to overcome the issues caused by the narrow genetic diversity in wheat production.

*Agropyron cristatum* exists as diploid (2n = 2x = 14, PP), tetraploid (2n = 4x = 28, PPPP) and hexaploid (2n = 6x = 42, PPPPPP) forms, all of which have excellent high yield phenotypes (multiple florets, multiple spikelets, excellent fertility), stress tolerance (drought tolerance, cold tolerance, salt tolerance) and disease resistance (powdery mildew resistance, stripe rust resistance, leaf rust resistance) (Dong et al. 2006; Zhou et al. 2018; Lin et al. 2022). In particularly, the 6P addition line 4844-12 (2n = 2x = 44) was generated by crossing the common wheat Fukuhokomugi (Fukuho) and *A. cristatum* (Z559), which resulted in a significant increase in GNS and TGW (Wu et al. 2006; Zhang et al. 2019). Then, a large number of translocation lines and deletion lines were obtained by radiation induction (Song et al. 2013). For example, PB260 and PB2978 with increased GNS, and PB3035, with a high TGW were generated in the Fukuho background (Zhang et al. 2018a; Zhang et al. 2016a; Zhang et al. 2015a). These translocation lines have been used to establish a bridge for transferring high-yield genes from *A. cristatum* into common wheat for genetic improvement. However, to date, no translocation lines have been reported that result in a simultaneous increase in both GNS and TGW for direct use in wheat breeding. Therefore, generating small segments of wheat–*A. cristatum* translocation lines with multi-grain number and high grain weight is valuable for breeding high-yielding wheat.

In the present study, we reported a stable wheat–*A. cristatum* 5BS·6PL translocation line WAT650I, which was identified among the lines derived from 4844-12 via cytological analysis, including genomic in situ hybridization (GISH), fluorescence in situ hybridization (FISH) and molecular marker analysis. Genetic population analysis showed that the segments from 4844-12 in WAT650I could simultaneously increase GNS and TGW. Furthermore, the locus responsible for increased GNS has been mapped to 6PL (16-17), and the locus responsible for high TGW has been mapped to 6PL (12-13) via a genetic population analysis of three translocation lines, namely, PB260, WAT642a and WAT650I. Therefore, this study provides valuable germplasm for the enrichment of wheat high-yield-related gene pools and their use in breeding programmes.

## Materials And Methods

### Plant materials

The wheat–*A. cristatum* disomic addition line 4844-12 (2n = 2x = 44) was derived from a cross between Fukuhokomugi (Fukuho; AABBDD, 2n = 6x = 42; from Japan) and *A. cristatum* (Z559; PPPP, 2n = 4x = 28; from Xinjiang, China). WAT650I was created by irradiating 4844-12 with $^{60}$Co-γ rays, followed by cross-pollination with Gaocheng8901. The $F_1$ progeny were backcrossed with Fukuho for three generations, and the homozygous translocation line WAT650I was obtained in the $BC_3F_2$ population with a Fukuho background. The $BC_4F_2$ and $BC_5F_2$ populations of WAT650I were used for genetic analysis. PB260 and del10c

were used as positive controls to identify the chromatin from *A. cristatum* in WAT650l (Song et al. 2016; Zhang et al. 2018a). In addition, PB260 and WAT642a were used to map the loci corresponding to increased the GNS and TGW (Zhang et al. 2018a; Song et al. 2013). All materials were planted in Xinxiang, Henan Province, during two growing seasons of 2020-2021 and 2021-2022. All the plant materials have been preserved at the Center of Crop Germplasm Resources Research at the Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China.

### GISH and FISH analysis

GISH was used to detect chromatin from *A. cristatum* (Z559) and to obtain the karyotypes of WAT650l. The seeds were soaked in $ddH_2O$ for approximately 24 hours in a petri dish until the seeds swelled and turned white, after which they were placed on moistened filter paper. The root tips of WAT650l were subjected to nitrous oxide and 90% acetic acid when the roots reached approximately 3~5 cm in length, and cells whose chromosomes were undergoing mitosis were obtained from the root tips by using a mixture of cellulase (R-10, Yakult, Japan) and pectinase (Y-23, Yakult Japan) for cell enzymatic hydrolysis (Wang et al. 2022b). Genomic DNA of Z559 was extracted using the modified cetyltrimethylammonium bromide (CTAB) method (Carra et al. 2007), diluted to approximately 200 ng/μl and labelled with Texas-red-5-dCTP (red). A similar procedure was performed according to the methods of Han et al. (2019).

The oligonucleotide probes Oligo-PTa535-1 (red) and Oligo-pSc119.2-1 (green) were used to detect wheat chromosomes by FISH analysis. The FISH procedure was performed according to the methods of Lin et al. (2022). The translocated chromosomes and possible mutations within Fukuho were determined by comparisons with the Chinese Spring karyotype (Tang et al. 2014). All the FISH probes used were synthesized by staff at Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The chromosome fluorescent signals were observed via an Olympus AX80 fluorescence microscope (Olympus Corporation, Tokyo, Japan) and imaged with a charge-coupled device (CCD) camera (Diagnostic Institute, Inc. Sterling Height, MI, USA).

### 6P-specific molecular marker genotyping for WAT650l

In a previous study, 6,063 *A.cristatum* P genome specific polymorphic expressed sequence tag-sequence tagged site (EST-STS) markers were developed in wheat backgrounds via transcriptome analysis (Zhang et al. 2015c). In addition, 255 6P-specific EST-STS markers were mapped to 31 physical regions of the 6P chromosome (Song et al. 2016). Here, a total of 36 6P-specific markers from 31 regions were selected for genotyping WAT650l.

Genomic DNA of all plant materials was extracted using a modified CTAB method (Carra et al. 2007). 5BS-specific markers were designed by Primer 5 based on 5BS terminal sequences obtained from the Unité de Recherche Génomique Info(URGI) (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v2.0/) database and sequences of wheat 5BS simple sequence repeat (SSR) markers obtained from the GrainGenes (https://wheat.pw.usda.gov/GG2/index.shtml) database. All the markers were synthesized by staff at Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Details of all the molecular markers used are shown in Table 1. PCR amplifications were carried out in a 20 μl, reaction mixture consisting of 10 μl of Green Master Mix, 1 μl of forwards primer (2 μM), 1 μl of reverse primer (2 μM), 1 μl of template DNA (80-100 ng/μl), and 7 μl of $ddH_2O$. Each PCR were as follows: one cycle at 94°C for 5 min for denaturation; 35 cycles at 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 then maintenance at 4°C. The PCR products were detected on 8% nondenaturing polyacrylamide gels.

### Investigation of agronomic traits and analysis of genetic populations

The following were planted at the Xinxiang experimental station of the Chinese Academy of Agricultural Sciences during the growing seasons of 2020-2021 and 2021-2022: homozygous translocation lines WAT650l, PB260 and WAT642a; disomic addition line 4844-12; recurrent parent Fukuho and Gaocheng8901; PB260 and WAT642a of the $BC_6F_2$ genetic population; and WAT650l of the $BC_4F_2$ and $BC_5F_2$ genetic populations. All these materials were planted such that the row spacing was 20 cm, that there were 18 plants in each row, and that the plants were spaced 10 cm apart. Flag leaf length was investigated at the heading stage. Plant height (PH), spike length, fertile tiller number, GNS, spikelet number per spike (SNS), kernel number per spikelet (KNS), TGW and weight per plant were evaluated after harvest. The repeat markers *Acpr3a* and *Acpr7* were used to determine the genotype of each plant of all genetic populations (Han et al. 2017). A *t* test was used to analyse the significance between positive and negative plants among the homozygous parents and all the genetic populations. GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) was used for data visualization.

## Results

### GISH and FISH analysis

GISH was used to analyse the number of chromosomes and to determine the karyotype of WAT650l using *A. cristatum* Z559 genomic DNA as GISH probe. The results indicated that WAT650l is a translocation line with 42 chromosomes. Among these, a pair of chromosomes had an obvious red signal at the terminus, and the other 40 chromosomes displayed completely blue 4,6-diamidino-2-phenylindole (DAPI) signals. These results confirmed that part of the *A. cristatum* chromatin was translocated into Fukuho(Fig. 1a).

To further verify the WAT650l karyotype, the oligonucleotide probes Oligo-PTa535-1 (red) and OligopSc119.2-1 (green) were used for FISH analysis. Compared with the Chinese Spring karyotype map, WAT650l had 14 complete chromosomes of the A subgenome and D subgenome, and 12 complete chromosomes of the B subgenome(1B, 2B, 3B, 4B, 6B, 7B) have been proven already. In addition, after combining the GISH results, we confirmed that the 5BS terminal segments of Fukuho translocated with the 6P chromosome of Z559 (Fig. 1b). In conclusion, WAT650l is a novel 5BS·6P small segment translocation line.

### Chromosomal location of introgressed chromatin in wheat–*A. cristatum* translocation line WAT650l

A total of 36 molecular markers from 31 segments of chromosome 6P were used to identify the segments from *A. cristatum* in WAT650l (Table 1). *A. cristatum* Z559, 4844-12, translocation line PB260 (harbouring 6PL14-17 bin) and deletion line del10c (harbouring 6PL1-13 bin) were used as positive controls, while Fukuho and Gaocheng 8901 were used as negative controls.

The results showed that all 36 markers could amplify specific bands in 4844-12. These bands were absent in the Fukuho and Gaocheng8901, and PB260 and del10c harbour bins 6PL (14-17) and 6PL (1-13), respectively, were confirmed in the same way as previously reported. All markers of the 6PL13 bin and two markers (*Agc10741* and *Agc68493*) of the 6PL12 bin amplified specific bands in WAT650l, but two markers, namely, *Agc9467* and *Agc22320*, from the 6PL12 bin and the other markers from the 6PS1-6PL11 bin failed to produce specific bands in WAT650l (Fig. 2a ~ 2i). Therefore, WAT650l harboured all the chromatin of the 6PL (13-17) bin and part of the chromatin of the 6PL12 bin. At the same time, three 5BS molecular markers, including two molecular markers designed using Chinese Spring reference genome sequences and the wheat-SSR marker *Xgwm234* were used to check the integrity of the 5BS chromosome in WAT650l (Table 1). Compared with the genotyping results of Fukuho and 4844-12, the three markers revealed no specific bands in WAT650l through polypropylene gel electrophoresis (Fig. 2j, 2k, 2l). These results further confirmed that chromosome 6PL (~12-17) bin of *A. cristatum* translocated with the 5BS terminal chromosome of Fukuho in WAT650l.

### Evaluation of WAT650l agronomic performance

The agronomic performance of WAT650l and its backcross parent Fukuho was investigated during the growing seasons of 2020-2021 and 2021-2022 in Xinxiang, Henan Province. The results showed that the average flag leaf length, plant height and spike length of WAT650l significantly increased by 4.59 cm, 16.73 cm, 1.14 cm, respectively, compared with those of Fukuho. In addition, through *t* test analysis, the GNS, KNS, SNS, and TGW of WAT650l increased by 14.46 and 11.94, by 1.26 and 0.66, by 2.41 and 4.67, and by 4.88 and 2.33, respectively, compared with those of Fukuho during the two years (*P* value < 0.01). However, there was a significant decrease in the number of effective tillers compared with the number for Fukuho (Table 2, Fig. 3a, 3b, 3f). The spike structure of WAT650l was also observed. We found that the WAT650l spikelets were more fertile, and most of the spikelets developed in balance on the whole, resulting in the spikes appearing 'stick'-shaped rather than 'spindle'-shaped like Fukuho (Fig. 3c, 3d, 3e).

To further verify the genetic benefits of 6PL terminal segments to the GNS and TGW of WAT650l, the yield traits (including effective tillers, weight per plant, KNS, GNS, SNS and TGW) of the plants composing the $BC_4F_2$ and $BC_5F_2$ populations were analysed. A total of 36 positive plants and 24 negative plants in the $BC_4F_2$ population and 108 positive plants and 93 negative plants in the $BC_5F_2$ populations were identified by the *A. cristatum* repeat sequence markers *Acpr3a* and *Acpr7*. Compared with negative plants, the positive plants in the $BC_4F_2$ population presented increases in SNS, KNS, GNS and TGW by 1.34, 0.33, 14.29 and 7.12, which were increases of 6.51%, 6.95%, 21.08% and 21.21%, respectively; the positive plants of the $BC_4F_2$ population presented a 3.15 decrease in the number of effective tillers, which was a decrease of 22.04% compared with that of the negative plants. Similarly, compared with negative plants, the positive plants in the $BC_5F_2$ population presented increases in SNS, KNS, GNS and TGW of 1.47, 0.55, 13.86 and 1.50, respectively, which were increases of 7.18%, 11.88%, 20.41% and 4.85%, respectively, and the positive plants of the $BC_5F_2$ generation presented a 2.32 decrease in the number of effective tillers, which was a decrease of 15.97% compared with that of the negative plants (*P* value < 0.01). However, there was no significant difference in weight per plant between the positive plants (17.47 g) and negative plants (19.21 g) in the $BC_5F_2$ genetic population (*P* value = 0.1022) (Table 2, Fig 4a, 4b). In addition, the segment from WAT650l was introgressed into KN199, the $BC_1F_1$ plants showed obvious high-yield traits, and the SNS, KNS, GNS and TGW increased by 2.18, 0.78, 11.75 and 11.25, respectively, which were increases of 10.71%, 18.48%, 19.19% and 30.44%, respectively (*P* value < 0.01) (Table 2). Therefore, the high-yield trait of WAT650l could stably inherited, and introgression of 6PL terminal segments from *A. cristatum* into Fukuho altered the spike development process and simultaneously increased the GNS and TGW.

### Chromosome mapping of the loci responsible for increased GNS and high TGW within the 6PL terminal region

Previously, a large number of 6P translocation lines and deletion lines carrying different-sized chromosomes were generated by radiation-induced mutations, and a locus responsible for increased GNS was located on 6PL (0.27-0.51) via deletion line mapping. In addition, some translocation lines carrying 6PL terminal segments showed increased GNS; this was the case for the 3BS·6PL (14-17) translocation line PB260 and the 3AL·6PL (16-17) translocation line WAT642a, which were generated previously (Fig. 5). Genetic population analysis confirmed that, compared with the negative plants, the positive plants in the PB260 $BC_6F_2$ population presented 0.29 and 6.55 increases in KNS and GNS, respectively, which were increases of 6.07% and 9.83%, respectively. Similarly, compared with the negative plants, the positive plants in the WAT642a $BC_6F_2$ population presented increases in KNS and GNS equal to 0.36 and 13.20, respectively, which were increases of 7.47% and 19.68%, respectively (*P* value < 0.01). However, no significant differences in SNS or TGW were observed between the positive plants and negative plants of the PB260 and WAT642a $BC_6F_2$ populations (Table 2, Fig. 4c, 4d). Taken together, these results showed that three translocation lines, PB260, WAT642a and WAT650l, carried the common segment from 6PL (16-17) and presented increased KNS and GNS (Fig. 6); on the other hand, the segment from 6PL (14-17) did not contributed to increased SNS and TGW. Therefore, the loci responsible for increased KNS and GNS were mapped to 6PL (16-17), and the loci corresponding to high SNS and TGW were mapped to 6PL (12-13) (Fig. 7).

## Discussion

### High-yield genes from *A. cristatum* are beneficial for wheat genetic improvement

Distant hybridization is an effective way to broaden gene pools for wheat breeding (Cheng et al. 2019; Li et al. 2019). With the successful introduction of many alien chromosomes into common wheat, many alien resistance genes have been widely used in wheat production, such as *Lr26/Sr31/Yr9/Pm8* of 1B·1R translocation lines of rye, *PM21* from *D. villosum*, and *Fhb7* from *Th. ponticum*; moreover, these genes have been used for breeding wheat resistance (Gao et al. 2020; Mater et al. 2004; Wang et al. 2020). Similarly, several wheat derived lines were generated via distant hybridization to increase GNS and TGW, the wheat–*D. villosum* T2VS·2DL translocation line NAU422 has longer spikes and produces more grains per spike, 6VS·6DL translocation lines in the Chinese

Spring background have been reported to increased TGW, and wheat– *Th. ponticum* 2A/2St and wheat– *Th. intermedium* 2B/2St disomic substitution lines in the common wheat background increased TGW (Zhang et al. 2015b; Feng et al. 2021; Wang et al. 2022a). However, a few high-yield alien genes are directly used for breeding high-yielding wheat today, because of the genetic burden and the negative relationship between GNS and TGW. In this case, *A. cristatum* has received extensive amounts of attention from breeders because of the agronomic traits of multiple flowers and spikelets. In recent years, 6P and 7P chromosome increase GNS and TGW were discovered, respectively (Wu et al. 2006; Sun et al. 2021), and many introgression lines, such as PB3504 and PB3228, that carry high-yield genes have been used for breeding high-yielding wheat(Chen et al. 2016; Liu et al. 2020). Furthermore, the use of several high-yield varieties, such as PB151 and Chuanmai93 has been promoted in recent years. Therefore, it is feasible to introduce high-yield genes from *A. cristatum* for breeding high-yield and super high-yield wheat. In this study, we developed a new translocation line, WAT650l, in which GNS and TGW simultaneously increased in the Fukuho background for the first time. In addition, the segments from WAT650l introduced into KN199 could also enhance GNS and TGW. Next, WAT650l will be used as a bridge to transfer high-yield genes to several main varieties for wheat genetic improvement.

### Translocation lines are effective tools for narrowing the genetic range

Due to the limitation of molecular marker density and the exchange rate between alien and wheat chromosomes, the traditional method of map-based cloning makes it difficult to precisely locate alien genes. Fortunately, the generation of deletion lines and translocation lines enables the narrowing of the genetic range. In our previous study, using deletion lines and translocation lines, we located many excellent genes from *A. cristatum*. For example, powdery mildew resistance genes and increased GNS genes were mapped to 6PL (0.27-0.51) by del27 and del16a (Lin et al. 2022; Zhang et al. 2019), leaf rust resistance genes and stripe rust resistance genes were mapped to 6PS (0.81-1.00) (Song et al. 2016; Zhang et al. 2017), and leaf rust resistance genes and powdery mildew resistance genes were mapped to 2PL (0.66-0.86) (Jiang et al. 2018; Li et al. 2017). Similarly, the blue-grain gene from *Th. ponticum* was successfully located on chromosome 4Ag (0.75-0.89) (Liu et al. 2018). In this study, three translocation lines, namely, PB260, WAT642a and WAT650l, that harbour different-sized segments were used to map the loci responsible for increased GNS and high TGW. Through genetic population analysis, the locus corresponding to increased GNS was mapped onto 6PL(16-17), and the locus corresponding to high TGW was mapped onto 6PL(12-13). Therefore, the use of translocation lines is effective for narrowing the genetic range and facilitating *A. cristatum* gene cloning in the future.

### WAT650l has great application value for wheat breeding

Alien genes can be easily introduced into wheat chromosomes in the form of addition lines, translocation lines and introgression lines. However, some alien chromosomes cannot be stably inherited in wheat. In addition, many materials cannot be directly used in wheat production due to genetic burdens (Jia et al. 2022). In this study, we generated and identified a small 5BS·6PL translocation line, WAT650l, whose translocated chromosome was confirmed to be stably inherited in the $M_2$, $M_3$ and $M_4$ generations via cytological analysis. At the same time, we found that the flag leaf length, plant height, spike length, SNS and KNS of WAT650l significantly increased and that the reduction in tiller number did not negatively affect the weight per plant. Notably, the GNS and TGW in the Fukuho background simultaneously increased during the two growing seasons. This is the first time that the terminal segment of the *A. cristatum* 6P chromosome introduced into wheat was shown to increase GNS and TGW simultaneously. Therefore, WAT650l is a valuable germplasm for using in wheat breeding. Next, we will work on transferring 6PL (12-17) from WAT650l to several main wheat varieties and further cloning the high-yield genes via resequencing.

## Declarations


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### Conflicts of interest/Competing interests

The authors declare no conflict of interest.

### Available of data and material

Data supporting the current study can be obtained by contacting the corresponding authors.

### Code availability

Not applicable

### Author's Contributions

LHL and SHZ conceived the research. YDL performed the research, analysed the data and wrote the paper. XZL, BH and JLY assisted in data collection. BJG, JPZ, HMH, WHL, XMY and XQL participated in the preparation of the reagents and materials in this study. All authors contributed to and approved the final manuscript.


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## Tables

Table 1 Molecular markers of 5BS and 6P chromosome.

| Markers | Forwards primer(5′-3′) | Reverse primer(5′-3′) | Annealing temperature (°C) | Bin |
|---|---|---|---|---|
| *AgC37790* | ACCTCGGCAGAGGAGCTT | CAACCAGTAGCAGGGGTGAG | 56 | 6PS14 |
| *AgC4282* | ACTGGCCTTCCTCTCTCTCC | GCACCCCAACAAAGAAAGAA | 58 | 6PS13 |
| *AgC5604* | GCAGCAAAATAGAGCTGCAA | TCCCAATGACCAACTTAACCA | 58 | 6PS12 |
| *AgC2861* | AGTCATATCAGCACTACTACTGCCA | GGCGTCGTTTTTTGTTTGTTG | 58 | 6PS11 |
| *AgC7094* | AGCAACACCATCACAATCATGTA | TGATTTGGGTAAGCAGAGGCT | 58 | 6PS10 |
| *AgC2637* | GGTGCTCGATCTGGAGCAACT | TGCCCTTGGATTCAGGAATC | 58 | 6PS9 |
| *AgC8950* | CAGATGAACAAGGTTCTGCCAT | TTCTCGCAAGGCATTTCAGA | 58 | 6PS8 |
| *AgC1368* | AAGCCCAGTACTACGAATCAGG | CGTGTGTGGGGATTTTTAGC | 56 | 6PS7 |
| *AgC3896* | AATGCAGGGCAATGGTAATC | AGTGCATGCATTTGTTGGAG | 56 | 6PS6 |
| *AgC10775* | AAGATCCTGACAACATGAAACC | ATGTAAAAACAAACCAAGGGG | 58 | 6PS5 |
| *AgC9835* | CAGCAGCAACACACTCACAA | CCAGAACAACCCCAATCTTC | 56 | 6PS4 |
| *AgC4527* | CGTGCTACCTCACAGGACAA | CCATCCCAAGTCAGGCTAGA | 56 | 6PS3 |
| *AgC9581* | TCCCTCTGTTGTATGTTGGCTAG | AACAAAGCAAGGTTGTTCCAGA | 56 | 6PS2 |
| *AgC7155* | CCTCTTTATATTTTCGGTGGTGA | CTGTTCCTGTGCCCCCG | 56 | 6PS1 |
| *AgC4399* | AATGTCTCGTCCTTTGCGGT | TGGAGGGGTGCCAAATGAG | 56 | 6PL1 |
| *AgC27067* | ATGGTTGGGATCCTTGACTTG | TCAAGCGTAATCTCAACTTGACC | 56 | 6PL2 |
| *AgC6900* | TTGGGTGATAAGAAGATGCCTC | AGTTGAGTGTGCTGCGATGC | 56 | 6PL3 |
| *AgC3466* | CGAAGCTTGGTATTGTCCAAAA | TCCAGCACGTGGAATTTGTC | 56 | 6PL4 |
| *AgC11930* | AAGGAAACTTCGGTGCTACG | TGTGTCCATGCCACTCAACT | 56 | 6PL5 |
| *AgC5385* | CTGACTTCCCATCACCGACT | TGGCCTTTCTTGAAGCATTT | 56 | 6PL6 |
| *AgC5103* | CAGTAACACTCCCTCCGATCC | TTGCACCGGTCTATCTCCTG | 56 | 6PL7 |
| *AgC15870* | CACATCCACCTGCAAGTCAG | CGGGGTCAGATGAAGTGAAT | 56 | 6PL8 |
| *AgC5532* | CAAATGCCGACACCGAGG | CCGGAACGCGTCGTAGAAC | 58 | 6PL9 |
| *AgC2453* | GGTGTTTTCCACTCTGTTGCA | TGCCAATAAAGGGCCCAATA | 58 | 6PL10 |
| *AgC32015* | GTGTAGTTCATGGGCGAAGC | CGTTGGTTTACGCTGGAGAT | 58 | 6PL11 |
| *AgC9467* | TGCAGCAATTGTCAACAACA | CTCTCTTCCCTGCAGCCTTA | 56 | 6PL12 |
| *AgC22320* | GCGGGGAGTCGTGGTAGTAGT | CGAAACGAGCAAGCTCTCC | 58 | 6PL12 |
| *AgC10741* | GTTATACCCGTAGGCGACGA | CGTTCGGACGGAGAAATATG | 56 | 6PL12 |
| *AgC68493* | CAGGTGGCTTCTTCGTCTTC | CATGAAGGAGGGTAAGGCTG | 56 | 6PL12 |
| *AgC7798* | GCCAAAGTCTCGAGCAAATC | GCTGTAGGCGAGGTTGTTTC | 56 | 6PL13 |
| *AgC9745* | AATGTCCCATCATGATGGAGG | TGATTCAGCTAGCCACTTGGAC | 58 | 6PL13 |
| *AgC21241* | TGCACGGATCTTCACAAAAG | GCTGCTTGTTCAAAACGTGA | 56 | 6PL13 |
| *AgC26719* | CAAGTGACAACTGCGGTCAT | TTCCTTACATAGTTGTTTCAGCAG | 56 | 6PL14 |
| *AgC11295* | GCCAGATTGTGCTGTTTCAA | GGATGCAGAAAACGAAGCTC | 58 | 6PL15 |
| *AgC12840* | CCCCAATAAGCTGCTCGTAG | ATCCACCGAGATAACCATCG | 58 | 6PL16 |
| *AgC4348* | AAGGAGGGAGTGAGAGAGAACA | GGGTCAAGGGAAGACTTCAGC | 58 | 6PL17 |
| *Xgwm234* | GAGTCCTGATGTGAAGCTGTTG | CTCATTGGGGTGTGTACGTG | 56 | 5BS |
| *5BS-1* | GAATCAGTGGCATTGGTATTAC | CCTCGGTGGACGAGACGAG | 56 | 5BS |
| *5BS-2* | GTAGTTGGCTCAGACCTT | CTGCGATTTCTTCATACA | 56 | 5BS |

Table 2 Significance analysis of agronomic traits of parents and populations.

| years | Materials | Generations | Plant number | Plant height | Spike length | Effective tillering | Spikelet number per spike | Kernel number per spielet | Grain number per spike |
|---|---|---|---|---|---|---|---|---|---|
| 2020-2021 | Gaocheng8901 | | 20 | 90.40±3.06 | 10.85±0.47** | 13.20±2.53** | 21.80±1.03 | 4.80±0.42 | 64.60±7.65 |
| | PB260 | M3 | 20 | 118.70±3.74** | 8.19±0.45 | 7.95±1.54 | 24.50±0.83** | 5.10±0.31** | 72.80±4.69** |
| | Fukuho | | 10 | 96.40±2.22 | 10.30±0.86 | 15.40±3.63** | 20.80±1.32 | 4.00±0.00 | 64.70±4.27 |
| | WAT642a | M3 | 20 | 91.63±3.11 | 9.61±0.6 | 10.37±2.69 | 21.37±0.9 | 5.11±0.57** | 81.32±9.24** |
| | WAT650l | M3 | 25 | 115.74±12.9** | 11.76±1.04** | 7.11±3.63 | 23.21±1.27** | 5.26±0.46** | 79.16±9.09** |
| | WAT650l | $BC_4F_2$/6P+ | 36 | 101.67±5.48** | 12.66±0.86** | 11.14±3.91 | 21.92±1.3** | 5.08±0.44** | 82.08±8.87** |
| | | $BC_4F_2$/6p- | 24 | 97.00±4.98 | 11.59±0.76 | 14.29±3.93** | 20.58±1.1 | 4.75±0.44 | 67.79±5.39 |
| 2021-2022 | Fukuho | | 20 | 87.90±1.97 | 10.85±0.47 | 16.20±2.90** | 18.20±0.63 | 4.60±0.52 | 64.10±4.48 |
| | WAT650l | M4 | 20 | 102.02±11.47** | 11.66±1.14** | 7.87±1.79 | 22.87±1.01** | 5.26±0.54** | 76.04±7.53** |
| | KN199 | | 20 | 83.41±2.42 | 9.93±0.62 | 11.22±2.37 | 20.35±0.88 | 4.22±0.42 | 61.22±8.42 |
| | KN199/WAT650l | | 20 | 121.06±6.12** | 11.51±0.76** | 12.16±2.99 | 22.53±0.62** | 5.00±0.44** | 72.97±9.13** |
| | PB260 | $BC_6F_2$/6P+ | 90 | 119.32±6.73 | 8.39±0.63 | 7.99±2.46 | 24.61±0.70 | 5.07±0.42** | 73.20±8.43** |
| | | $BC_6F_2$/6P- | 60 | 119.60±4.96 | 8.07±0.48 | 8.03±2.12 | 24.55±0.81 | 4.78±0.52 | 66.65±8.92 |
| | WAT642a | $BC_6F_2$/6P+ | 99 | 93.67±4.25 | 9.61±0.7 | 11.06±2.76 | 21.27±0.78 | 5.18±0.53** | 80.29±9.83** |
| | | $BC_6F_2$/6P- | 21 | 93.00±2.93 | 9.10±0.61 | 8.18±1.89 | 21.18±0.60 | 4.82±0.40 | 67.09±4.32 |
| | WAT650l | $BC_5F_2$/6P+ | 108 | 101.01±5.55** | 12.72±0.81** | 11.21±3.40 | 21.94±1.13** | 5.18±0.47** | 81.77±8.93** |
| | | $BC_5F_2$/6p- | 93 | 98.09±5.94 | 11.62±0.73 | 14.53±4.76** | 20.47±1.35 | 4.63±0.51 | 67.91±7.60 |

* and **denoted significant differences by *t* test at the probability levels of *P* value = 0.05 and *P* value = 0.01.

## Figures

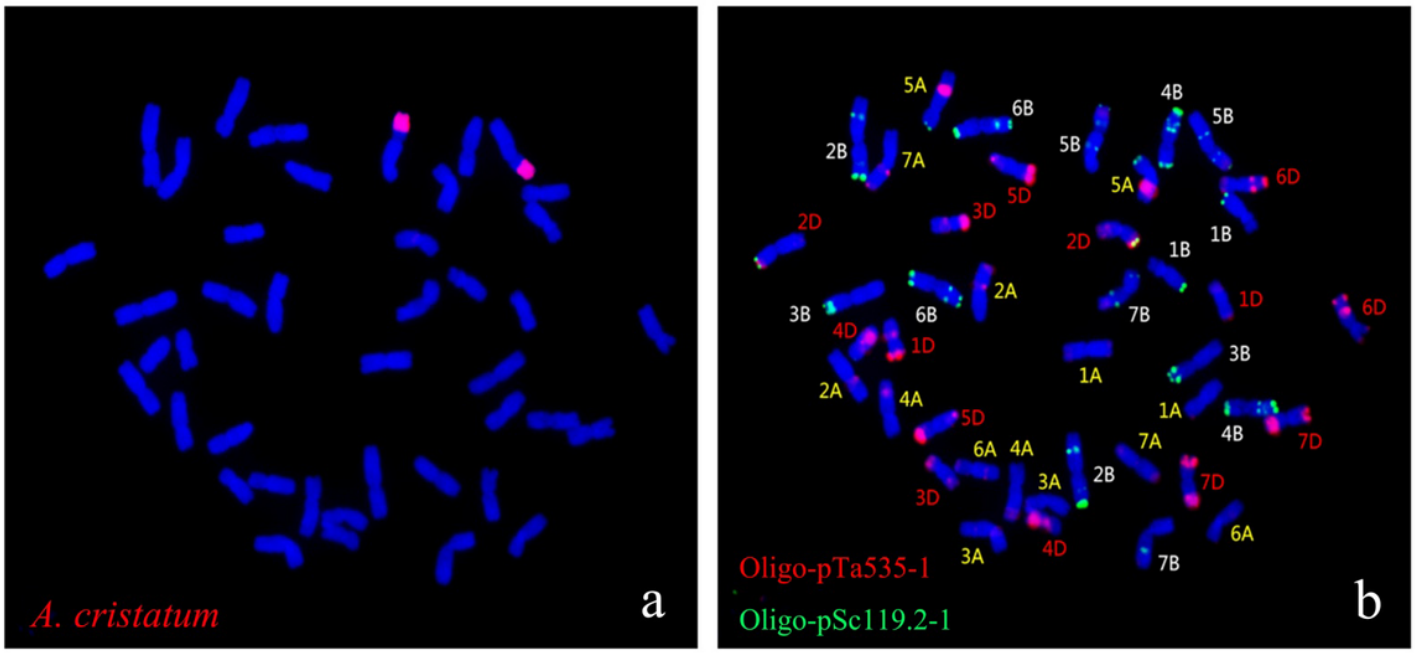


Figure 1

GISH (a) and FISH (b) analysis of WAT650I. Chromosomes were counterstained with DAPI and visualized with blue fluorescence.

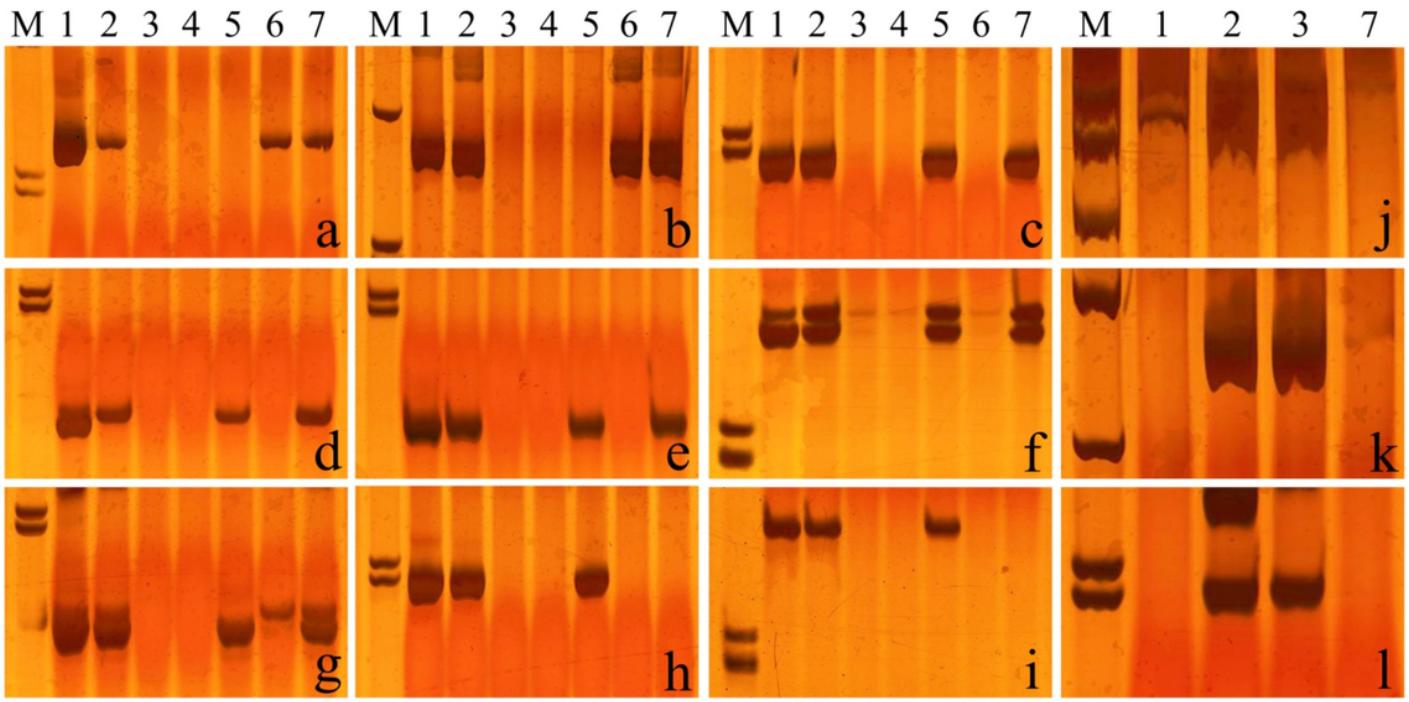


Figure 2

Partial 6P-specific EST-STS molecular marker analysis. M: Marker D2000; 1: Z559; 2: 4844-12; 3: Fukuho; 4: Gaocheng8901; 5: del10c; 6: PB260; 7: WAT650l. a: *Agc11295*; b: *Agc26719*; c: *Agc7798*; d: *Agc21241*; e: *Agc9745*; f: *Agc68493*; g: *Agc10741*; h: *Agc9467*; i: *Agc22320*; j: 5b1; k: 5b2; l: *Xgwm234*

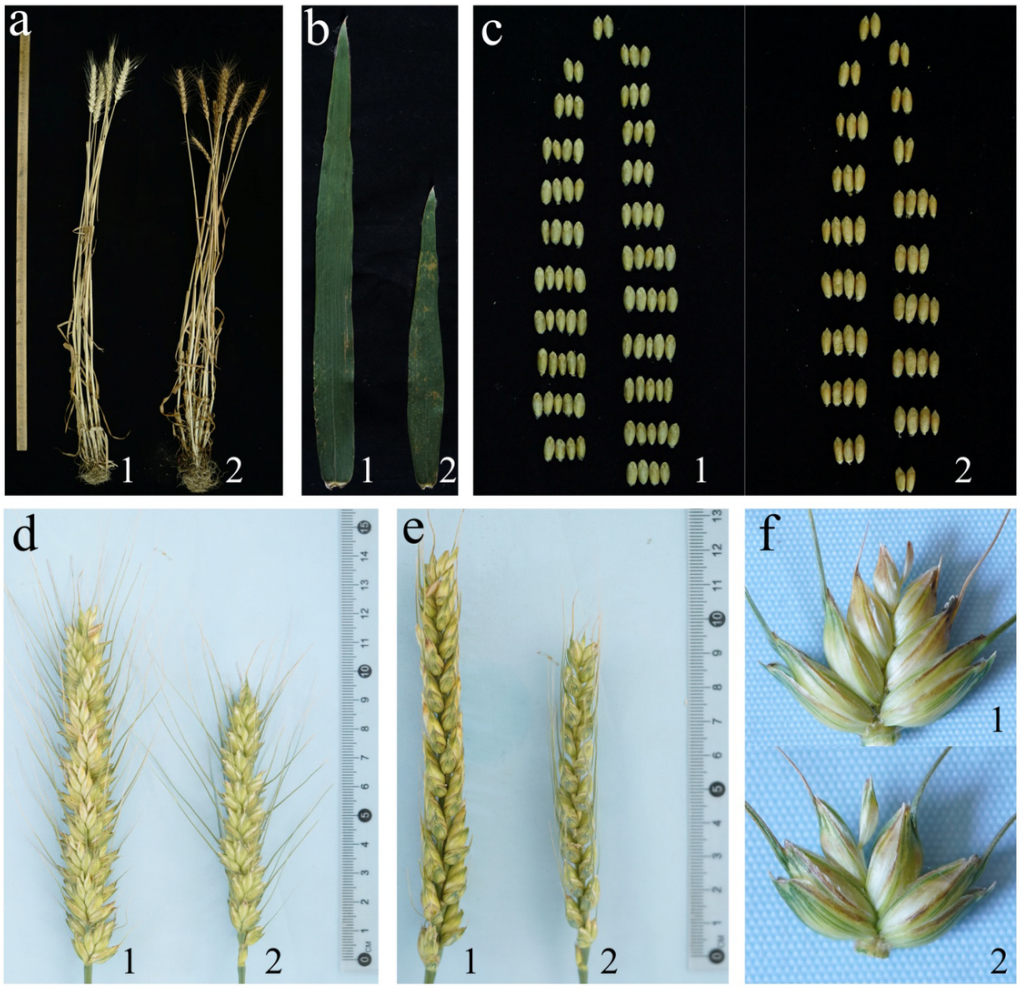


**Figure 3**

Assessment of agronomic traits of WAT650l and Fukuho. a: plant at adult stage. b: flag leaf. c: spike structure. d and e: the major spike. f: spikelet. 1: WAT650l; 2: Fukuho.

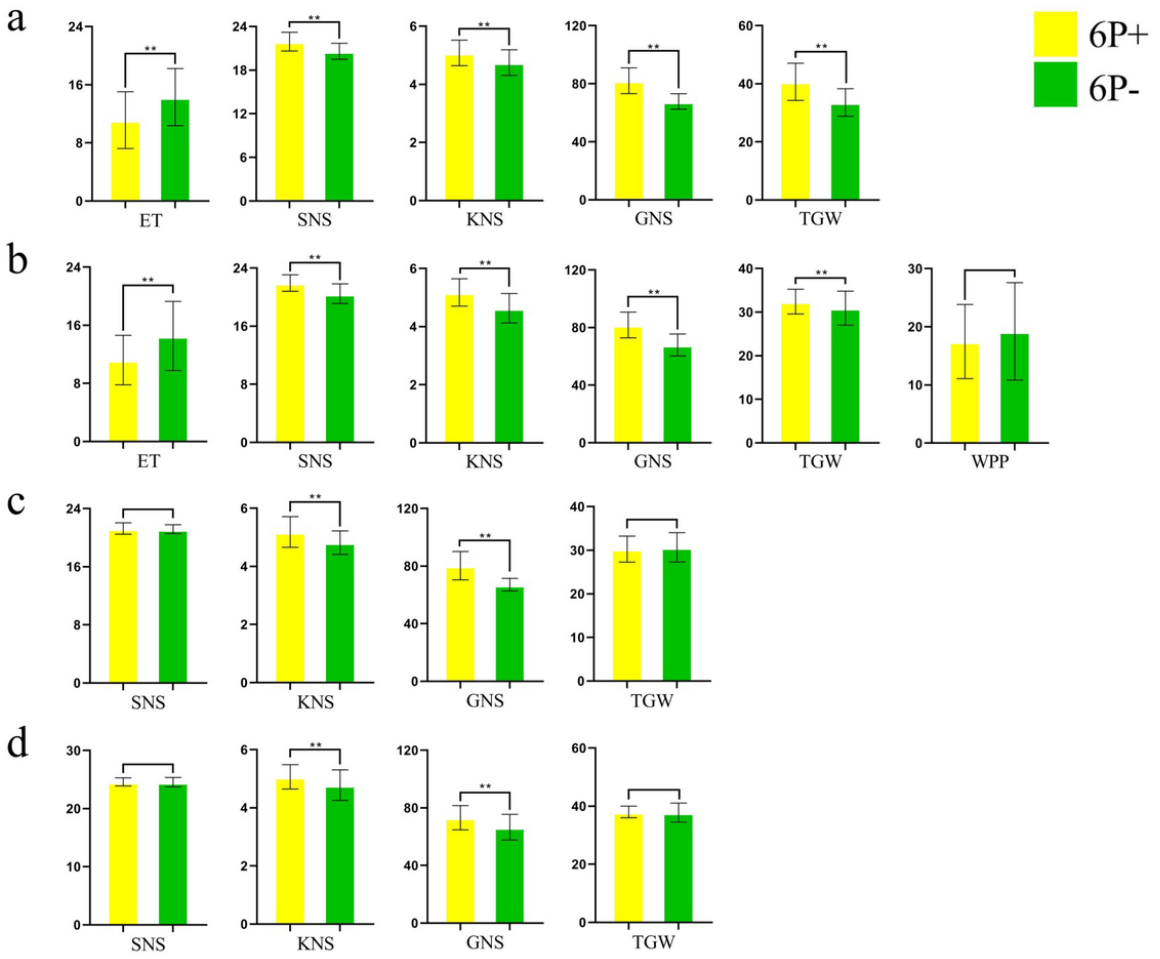


**Figure 4**

Significance analysis of agronomic traits. a: significance analysis of WAT650l BC4F2population about effective tillers(ET), SNS, KNS, GNS and TGW in 2020-2021. b: significance analysis of WAT650l BC5F2population about ET, weight per plant (WPP), SNS, KNS, GNS and TGW in 2021-2022. c: significance analysis of WAT642a BC6F2 population about KNS, SNS, GNS and TGW. d: significance analysis of PB260 BC6F2population about KNS, SNS, GNS and TGW.

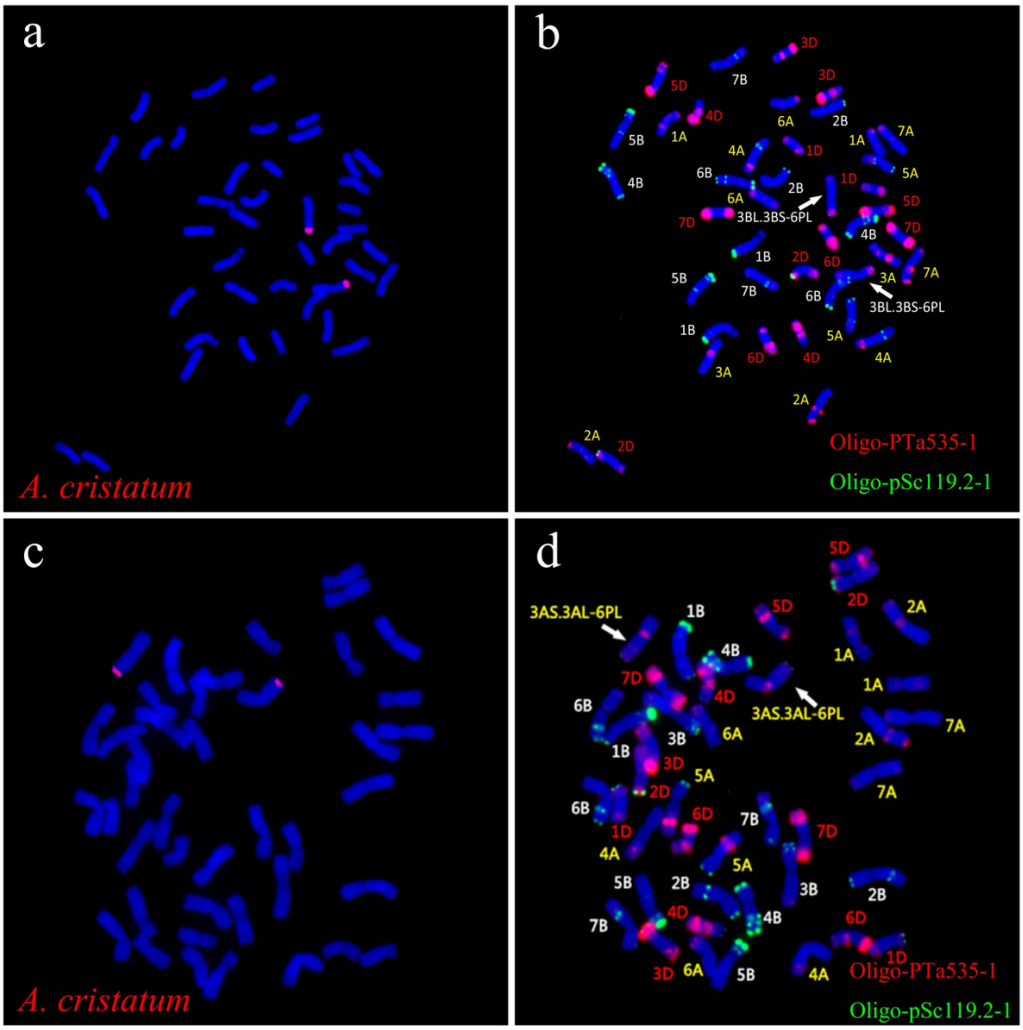


**Figure 5**

GISH and FISH analysis of PB260 and WAT642a. a: GISH analysis of PB260; b:  FISH analysis of PB260; c: GISH analysis of WAT642a; d: FISH analysis of WAT642a.

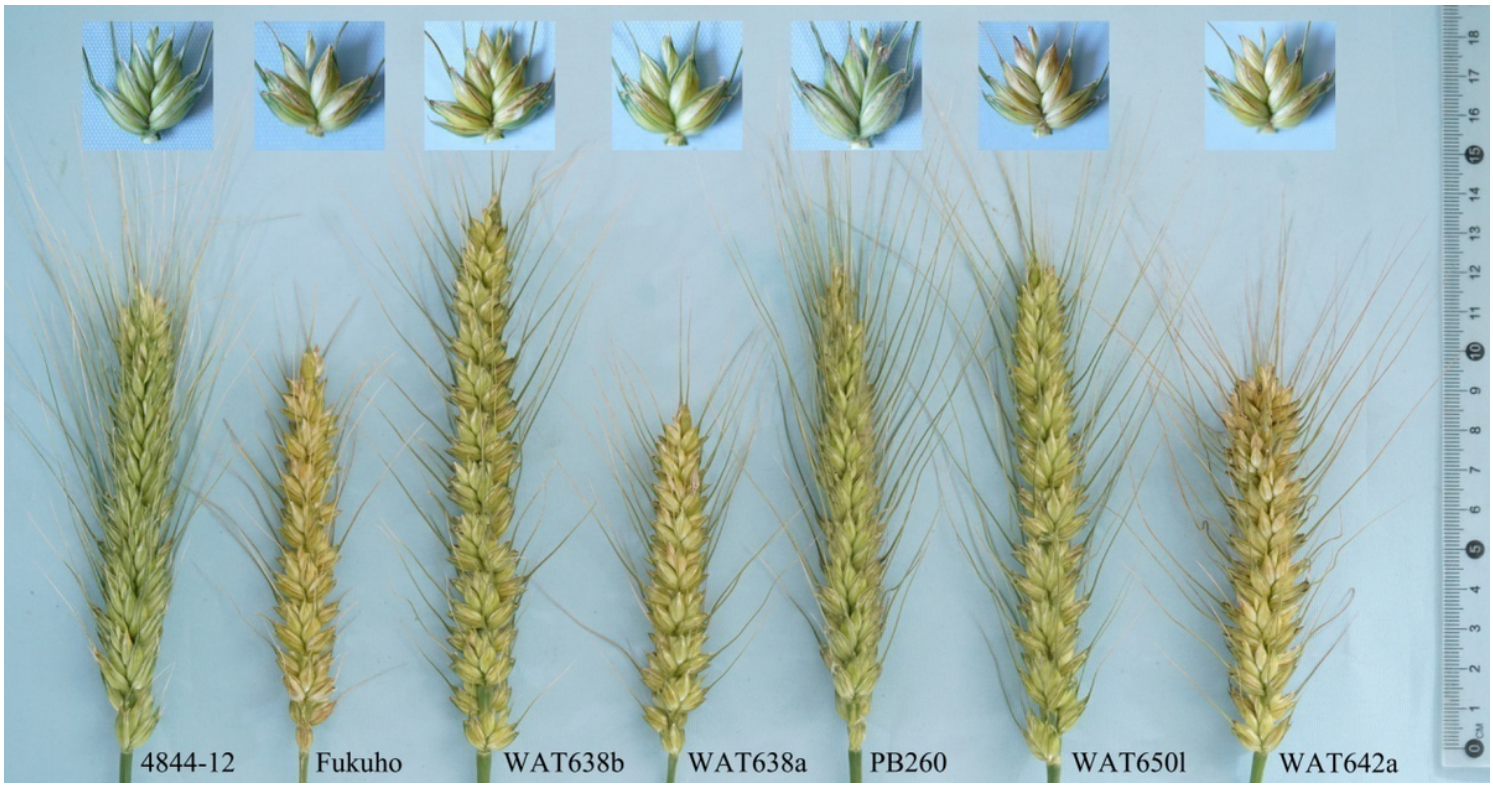


Figure 6

Evaluation of the spike straits of chromosome 6P addition line 4844-12, Fukuho, WAT638b, WAT638a, PB260, WAT650l and WAT642a.

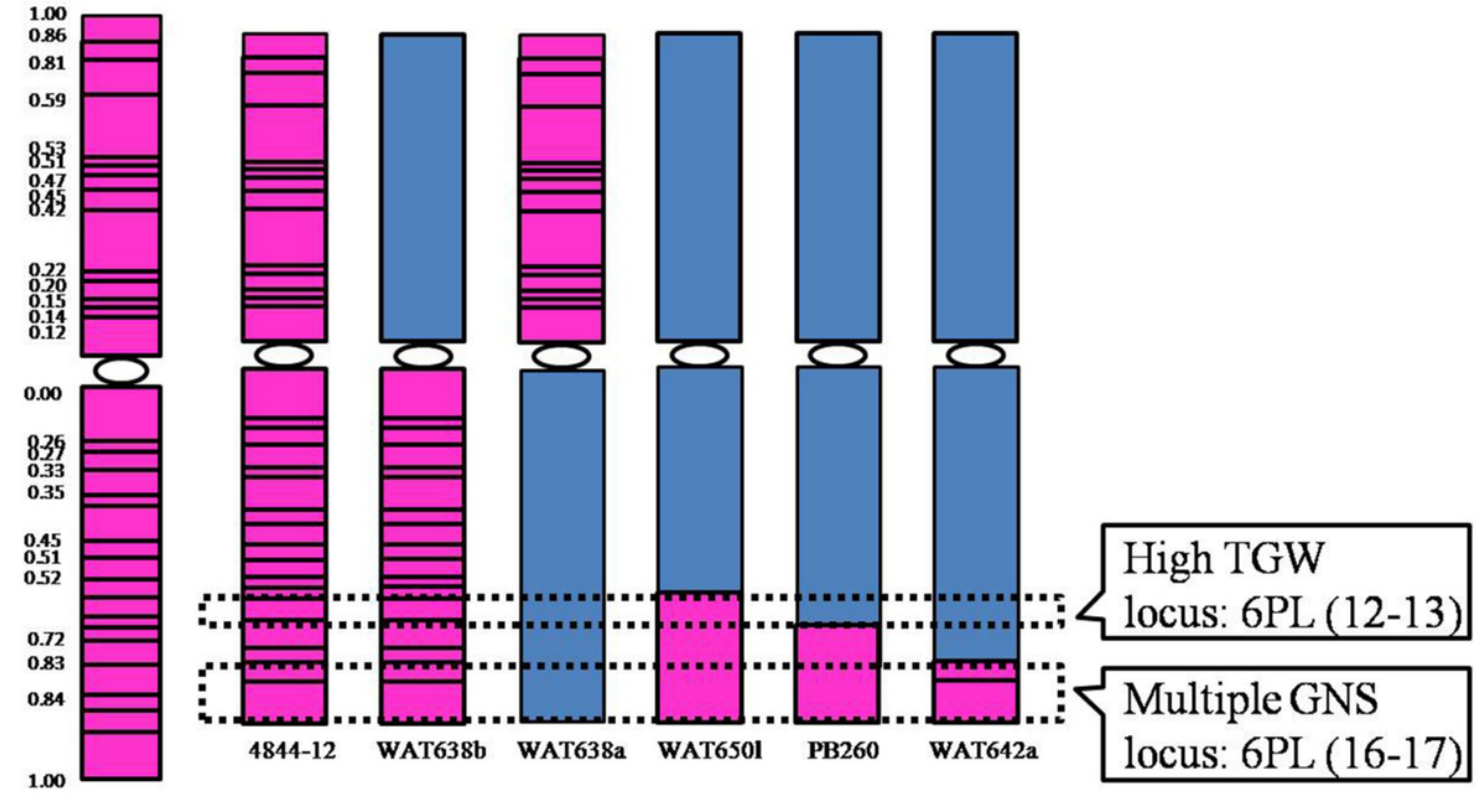


Figure 7

Chromosome diagrams of the locus of the GNS and TGW. On the left is 31 chromosome loci of 6P. Pink represents *A. cristatum* chromatin, blue represents Fukuho chromatin.