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Ziyuan Kuang
CAAS: Chinese Academy of Agricultural Sciences
Xiajie Ji
CAAS: Chinese Academy of Agricultural Sciences
Shirui Xu
CAAS: Chinese Academy of Agricultural Sciences
Haiming Han
CAAS: Chinese Academy of Agricultural Sciences
Jinpeng Zhang
CAAS: Chinese Academy of Agricultural Sciences
Shenghui Zhou
CAAS: Chinese Academy of Agricultural Sciences
Xinming Yang
CAAS: Chinese Academy of Agricultural Sciences
Xiuquan Li
CAAS: Chinese Academy of Agricultural Sciences
Lihui Li
CAAS: Chinese Academy of Agricultural Sciences https://orcid.org/0000-0001-6907-0638
Weihua Liu (liuweihua@caas.cn)
CAAS: Chinese Academy of Agricultural Sciences https://orcid.org/0000-0002-8527-9973

Research Article

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Generation and identification of a wheat–*Agropyron cristatum* (L.) Gaertn. 3P chromosome addition line and substitution line

Ziyuan Kuang [1], Xiajie Ji [1], Shirui Xu [1], Haiming Han [1], Jinpeng Zhang [1], Shenghui Zhou [1], Xinming Yang [1], Xiuquan Li [1], Lihui Li [1,*] and Weihua Liu [1,*]

[1] National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Sciences,Chinese Academy of Agricultural Sciences, Beijing 100081, China; 18853870968@163.com (K.Z.); jixiajie003@126.com (X.J.); xushirui1105@163.com (S.X.); hanhaiming@caas.cn (H.H.); zhangjinpeng@caas.cn (J.Z.); zhoushenghui@caas.cn (S.Z.); yangxinming@caas.cn (X.Y.); lixiuquan@caas.cn (X.L.)

[*] Correspondence: lilihui@caas.cn (L.L.); liuweihua@caas.cn (W.L.)

Abstract

Agropyron cristatum (L.) Gaertn. (2n=4x=28, PPPP), which is a wild relative of common wheat and has a P genome, has many excellent characteristics (for example, it is disease resistant, is stress resistant and is high yielding) and has important utilization value for the genetic improvement of wheat. In this study, the wheat–*A. cristatum* 3P addition line I-27 and 3P(3B) substitution line I-15 were identified for the first time via in situ hybridization (ISH) and specific molecular marker (EST-STS) from wheat–*A. cristatum*-derived lines, which laid an representing important material for the utilization of excellent genes from *A. cristatum* chromosome 3P for wheat genetic improvement. In terms of agronomic traits, that the tiller number and spike length of 3P addition line I-27 were significantly higher than those of Fukuhokomugi. The spike length and spikelet number per spike of 3P substitution line I-15 were significantly greater than those of Fukuho. Additionally, wheat–*A. cristatum* 3P substitution line I-15 was resistant to wheat leaf rust. The generation of the 3P addition line I-27 and 3P substitution line I-15 was essential for the transfer of the excellent genes of the *A. cristatum* 3P chromosome into common wheat and resulted in the wheat–*A. cristatum* addition line containing a complete set of genetic research materials, laying a foundation for comparative studies of the P genome and subsequent fine mapping.

Keywords wheat, *Agropyron cristatum*, 3P addition line, 3P substitution line, leaf rust

Introduction

Wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD) is one of the most important crop species and is widely grown worldwide. Moreover, wheat plays an important role in grain production in China and worldwide. In recent years, the demand for wheat has grown throughout the world (Hunter et al. 2017). Owing to the large-scale cultivation of wheat cultivars, the genetic basis of wheat cultivars is becoming increasingly narrow, and the improvement of wheat cultivars is limited (Sun et al. 2021). Wild relatives of common wheat serve as excellent gene libraries for wheat genetic improvement. Introducing excellent genes into common cultivated wheat could constitute an effective strategy for broadening the genetic basis of wheat and for wheat genetic improvement.

Wild relatives of wheat, especially tertiary gene sources, have a large number of excellent genes, such as those that provide strong tolerance to biotic and abiotic stresses, and thus are important materials for the genetic improvement of common wheat (Li et al. 2020; Zhou et al. 2020). Many wild relatives of wheat have been successfully crossed with wheat, and a large number of alien addition lines and substitution lines have been obtained (Chapman and Riley, 1955; Friebe et al.1992; Chen et al.1995; Li et al. 1997, 1998; Chen et al. 2005; Schneider et al. 2005; Li and Wang 2009). Therefore, exogenous genes from wheat relatives can be introduced into common wheat via distant hybridization and chromosome engineering, and genetic improvement of wheat can be realized. With multiple spikelets and resistance to powdery mildew, the wheat–rye 1R addition line was obtained from a cross of Shaanmai 611 and Austrian rye (Yang et al. 2016). Zhang et al. (2021) obtained a new wheat variety with resistance to powdery mildew through a wheat–*Dasypyrum villosum* 1V(1D) substitution line. Yang et al. (2017) demonstrated that homoeologous group 3 chromosomes of *Leymus mollis* contain a new stripe rust resistance gene according to different wheat–*L. mollis* double monosomic addition lines. A major Fusarium head blight (FHB) resistance gene, *Fhb7*, was found and designated in the wheat–*Thinopyrum ponticum* 7E(7D) substitution line, and the *Fhb7* gene has been cloned successfully by map-based cloning (Guo et al. 2015; Wang et al. 2020). Therefore, the discovery and utilization of exogenous excellent genes provides more extensive germplasm selection for breeders.

Agropyron cristatum (L.) Gaertn (2n=4x=28, PPPP) is an important wild relative of common wheat (Dewey 1969, 1984); has high economic value, contains a large number of desirable genes that can be exploited for wheat improvement, such as genes that provide resistance to powdery mildew, resistance to stripe rust, tolerance to drought, tolerance to cold and increased yield; and is vitally important for wheat improvement (Liu et al. 2010; Li et al. 2016; Jiang et al.2018). By using wide hybridization and embryo rescue, Li et al. (1997, 1998) successfully obtained intergeneric hybrids between *A. cristatum* and common wheat, and then a series of wheat–*A. cristatum* addition lines were obtained from wheat–*A. cristatum*-derived lines, laying a foundation for wheat improvement by the use of these desirable genes. Among the exogenous addition lines and substitution lines containing the *A. cristatum* P chromosome that have been identified thus far, the 1P chromosome carries genes related to abiotic stress resistance and improved plant architecture (Pan et al. 2017; Wang et al. 2022); the 2P chromosome carries powdery mildew and leaf rust resistance genes (Li et al. 2016; Jiang et al. 2018);

the 5P chromosome can promote partial homoeologous chromosome recombination (Pan et al. 2022); the 6P chromosome can increase wheat yields by promoting the number of fertile spikelets per spike and final kernel number per spike (Wu et al. 2006; Ye et al. 2015); and the 7P chromosome carries grain weight-related gene(s) that positively regulate yield traits (Sun et al. 2021). However, the excellent genes of the wheat–*A. cristatum* 3P addition line and *A. cristatum* 3P chromosomes have not been reported.

In this study, wheat–*A. cristatum* 3P addition line I-27 and 3P-3B substitution line I-15 were identified from among wheat–*A. cristatum*-derived lines, and the chromosome composition and agronomic traits of the two materials were characterized. Additionally, we developed 3P-specific molecular markers to track *A. cristatum* 3P chromatin, laying a foundation for the subsequent exploration and utilization of excellent traits controlled by the 3P chromosome.

Materials and Methods

Plant Materials

Wheat–*A. cristatum*-derived lines I-27 and I-15 were derived from a cross between *A. cristatum* accession Z559 (2n=4x=28, PPPP) and *T. aestivum* cv. Fukuhokomugi (Fukuho) (2n=6x=42, AABBDD). The following wheat–*A. cristatum* disomic addition lines were used: II-3-1a (Pan et al. 2017), II-9-3 (Li et al., 2016), II-21-2 (Liu et al., 2010), II-11-1b (Pan et al. 2022), 4844-12 (Wu et al., 2006), and II-5-1 (Lu et al., 2016). All these materials were provided by the Center of Crop Germplasm Resources Research at the Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China.

Molecular Marker Analysis

Thirty-four pairs of universal molecular markers of the *A. cristatum* P genome were used to identify the exogenous P chromosome, and 42 pairs of expressed sequence tag-sequence tagged site (EST-STS) markers for different P chromosomes were used to identify partial homoeologous groups of alien chromosomes (Table S1) (Han et al. 2017; Zhang et al. 2017). The tetraploid *A. cristatum* genome reference sequence was compared to the wheat reference sequence IWGSC RefSeq v2.1 by BLASTN queries of site sequence differences to design 3P-specific molecular markers. The 3P-specific molecular marker primer sequences are shown in Table S2. The PCR amplification procedure and product analysis were performed as described by Zhang et al. (2017).

Cytological Detection

To determine the genetic composition of wheat–*A. cristatum* I-27 and I-15, the root tip cells at mitotic metaphase of and pollen mother cells (PMCs) at metaphase I were observed. Mitotic and meiotic cells were observed according to previous methods (Jiang et al 2016; Li et al. 2020).

Genomic in situ Hybridization (GISH) and Fluorescence in situ Hybridization (FISH)

The exogenous chromosomes of wheat–*A. cristatum* I-27 and I-15 were detected via GISH. Genomic DNA of *A. cristatum* accession Z559 was used as a probe, and genomic DNA of Fukuho was used as a blocker to confirm the presence of *A. cristatum* chromosomes in the wheat background (Luan et al. 2010). The repeat sequences oligo-pTa535-1 and oligo-pSc119.2-1 were used to distinguish wheat chromosomes (Tang et al. 2014), and the repeat sequences pAcTRT1 and pAcpCR2 were used to distinguish *A. cristatum* chromosomes via FISH (Han et al. 2019). GISH and FISH experiments were carried out according to previous methods, with slight modifications (Liu et al. 2010; Sun et al. 2021). All the hybridization signals were observed by a Zeiss Axio Imager Z2 upright epifluorescence microscope (Carl Zeiss, Ltd, Germany), captured with a charge-coupled device (CCD) camera and analyzed by ISIS software (MetaSystems GmbH, Germany).

Identification of Leaf Rust Resistance at the Adult Stage

In this study, the materials were inoculated with mixtures of THT and PHT strains at the jointing stage by the spray method, and the common wheat cultivar Zhengzhou 5389 was used as a susceptible control. The resistance to leaf rust at the adult stage in the field was assessed in accordance with the methods of Jiang et al. (2018). A 0 to 4 scale was used for ITs, as described by Roelfs (1992).

Evaluation of Agronomic Traits

The wheat–*A. cristatum*-derived lines I-27 and I-15 and wheat cultivar Fukuho were planted at the experimental farm of the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Xinxiang, Henan Province, during the 2019-2020 and 2020-2021 growing seasons. The row length was 2.0 m, and the row spacing was 30 cm; each row contained 20 seeds (Pan et al. 2022). Plant height, spike length, fertile tiller number, spikelet number, spikelet number per spike and grain number per spike were evaluated for each individual plant. All the data were analyzed by SPSS 25.0 (SPSS, Chicago, IL, USA).

Results

Molecular Marker Analysis of Wheat–*A. cristatum*-Derived Lines

To clarify partial homoeologous groups of the P chromosomes in the wheat–*A. cristatum*-derived lines, 70 wheat–*A. cristatum*-derived lines were screened by using the universal molecular markers of the P genome, after which EST-STS markers for different P chromosomes and 3P-specific molecular markers were used to identify the derived lines that contained the P genome. The results showed that wheat–*A. cristatum*-derived lines I-27 and I-15 had amplification bands corresponding to the P genome molecular markers and 3P-specific molecular markers, while the other P chromosome molecular markers did not show amplification bands (Fig. 1). This indicates that the wheat–*A. cristatum*-derived lines I-27 and I-15 carry the homoeologous group 3 chromosome of *A. cristatum*, namely, those wheat–*A. cristatum*-derived lines with chromosome 3P.

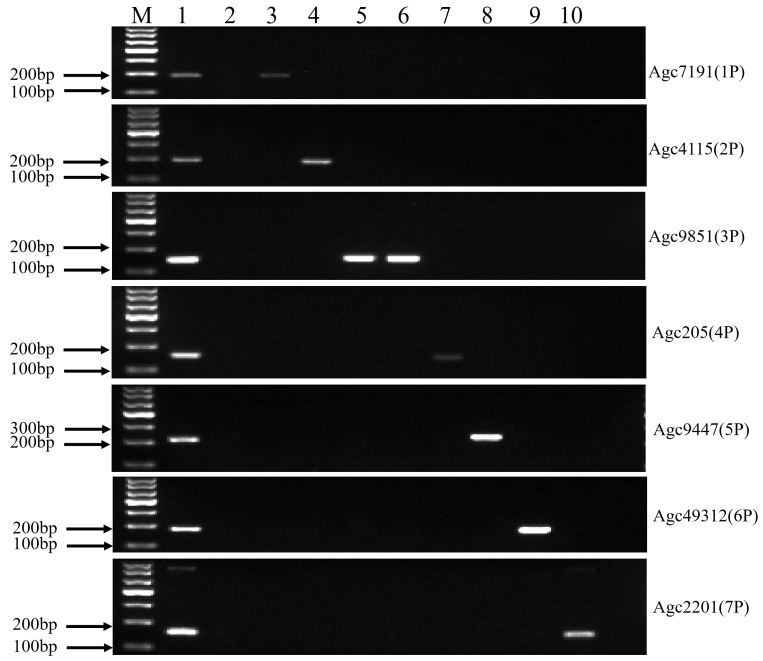

Fig. 1 Identification of 1~7P-specific molecular markers of wheat–*A. cristatum*-derived lines

M: DNA marker, 1: *A. cristatum* accession Z559, 2: Fukuho common wheat, 3: II-3-1a (wheat–*A. cristatum* 1P addition line), 4: II-9-3 (wheat–*A. cristatum* 2P addition line), 5: I-27, 6: I-15, 7: II-21-2 (wheat–*A. cristatum* 4P addition line), 8: II-11-1b (wheat–*A. cristatum* 5P addition line), 9: 4844-12 (wheat–*A. cristatum* 6P addition line), 10: II-5-1 (wheat–*A. cristatum* 7P addition line). The top-down sequence on the right side is the specific primer name for the 1-7 P homoeologous groups.

Cytological Identification and GISH/FISH Analysis of Wheat–*A. cristatum*-Derived Lines I-27 and I-15

The probes pAcTRT1 and pAcpCR2 were used to distinguish among the homoeologous groups of *A. cristatum* chromosomes in derived lines I-27 and I-15. The results showed that both wheat–*A. cristatum*-derived lines I-27 and I-15 contained two *A. cristatum* 3P chromosomes (Fig. 2c, 2g). The number of I-27 chromosomes was 2n=22II, with an average of 0.35 univalents, 1.87 rod bivalents and 19.85 ring bivalents (Table 1); included were 42 wheat chromosomes and two *A. cristatum* 3P chromosomes (Fig. 2a, 2b, Fig. 3a). According to the GISH results of PMCs at metaphase I, wheat–*A. cristatum*-derived line I-27 possessed a paird ring bivalent which were two 3P chromosomes with red signal (Fig. 2d), indicating that derived line I-27 was a wheat–*A. cristatum* 3P disomic addition line. Similarly, the chromosome number of wheat–*A. cristatum*-derived line I-15 was 2n=21II, with an average of 0.32 univalents, 1.38 rod bivalents and 19.37 ring bivalents (Table 1). I-15 thus contained 40 wheat chromosomes and a pair of 3P chromosomes (Fig. 2e, 2f, 2h). Unlike with the karyotype of Chinese Spring common wheat revealed by FISH signals (Tang et al. 2014b), a pair of common wheat 3B chromosomes were replaced by a pair of 3P chromosomes (Fig. 3b). The above results showed that derived line I-27 was a wheat–*A. cristatum* 3P(3B) disomic substitution line.

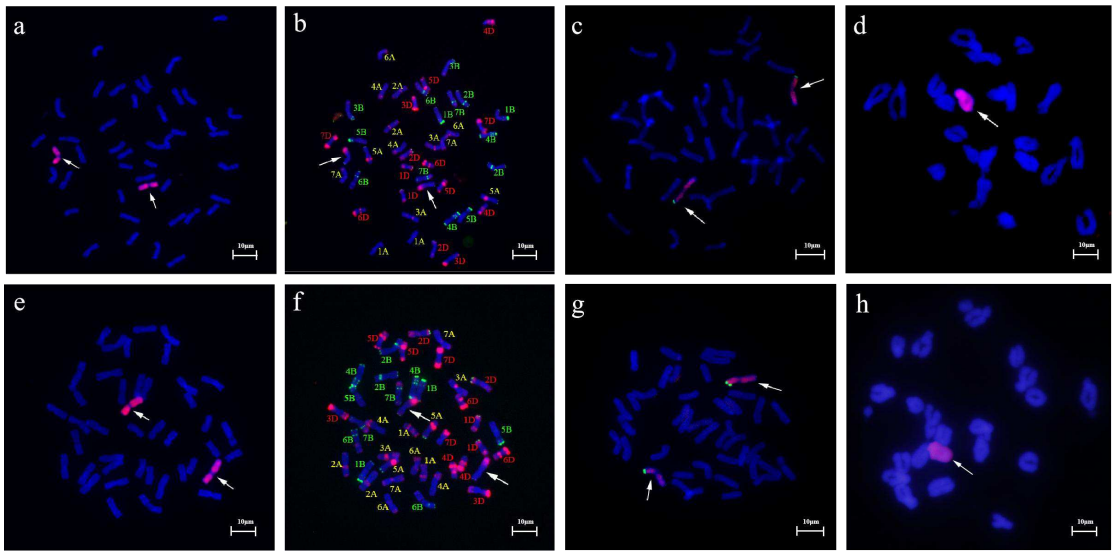

Fig. 2 Mitotic and meiotic GISH/FISH of wheat–*A. cristatum*-derived lines I-27 and I-15

(a-d) Cells derived from wheat–*A. cristatum*-derived line I-27, **(e-h)** Cells derived from wheat–*A. cristatum*-derived line I-15. **a**, **e**: Mitotic GISH analysis of wheat–*A. cristatum*-derived lines I-27(a) and I-15(e). The wheat chromosome was restained blue by 4',6-diamidino-2-phenylindole (DAPI) and the red signal indicates the *A. cristatum* chromosome. **b**, **f**: Mitotic FISH analysis of wheat–*A. cristatum*-derived lines I-27(b) and I-15(f). The arrow refers to the *A. cristatum* chromosome, and wheat chromosomes were restained blue by DAPI. The repeat sequence probe Oligo-pSc119.2 is labeled by the green signal, and the repeat sequence probe Oligo-pTa535 is labeled by the red signal. **c**, **g** The repeat sequence probe pAcTRT1 is labeled green, and the repeat sequence probe pAcpCR2 is labeled red. Wheat chromosomes were restained blue by DAPI. **d**, **h**: Meiotic GISH analysis of wheat–*A. cristatum*-derived lines I-27(d) and I-15(h). The wheat chromosome was restained blue by DAPI, and the red signal indicates the *A. cristatum* chromosome.

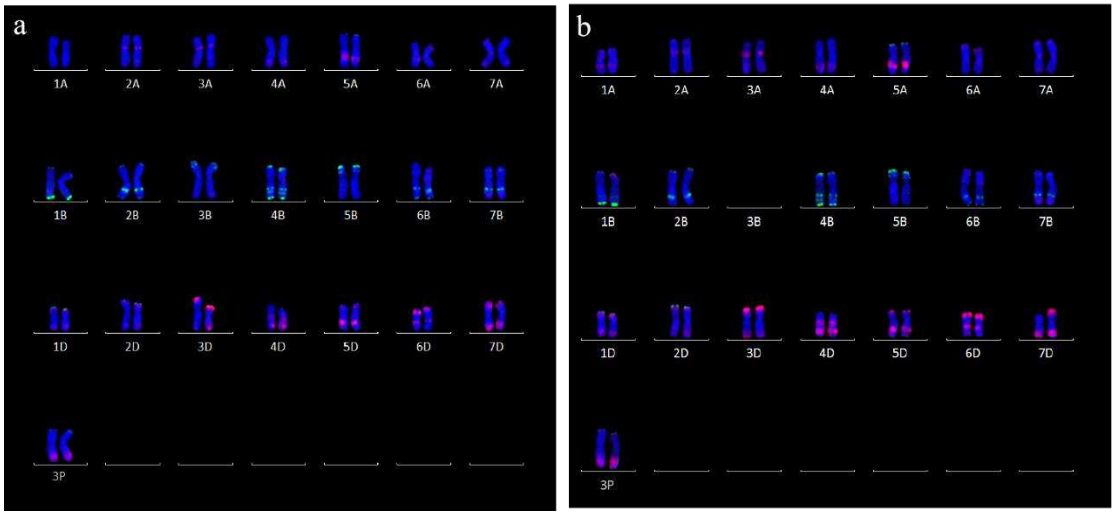

Fig. 3 Karyotypes of I-27 (a) and I-15 (b) based on FISH assays

177 **Table 1** Chromosome number and morphological identification of PMCs at metaphase I of meiosis in three
178 materials

Materials	2n	No. of cells	Chromosome configuration			
			Univalent	Bivalent		
				Rod	Ring	Total
I-27	44	100	0.35 (0-2)	1.87 (0-2)	19.85 (19-22)	21.72 (21-22)
I-15	42	100	0.32 (0-2)	1.38 (0-2)	19.37 (19-21)	20.75 (20-21)
Fukuho	42	100	0.06 (0-2)	1.32 (0-3)	19.64 (18-21)	20.96 (20-21)

179 **Agronomic Trait Evaluation and Leaf Rust Resistance Identification**

180 To evaluate the characteristics of the wheat–*A. cristatum* 3P addition line I-27 and substitution line
181 I-15 and their value for wheat breeding and disease resistance research, the agronomic traits of the two
182 materials were evaluated. Compared with those of Fukuho, the plant height, fertile tiller number and
183 spike length of 3P addition line I-27 were significantly increased(Table 2, Fig. 4). Similarly, the plant
184 height, spike length and spikelet number per spike of 3P substitution line I-15 were significantly higher
185 than those of Fukuho (Table 2, Fig. 4). The results showed that the recurrent parent Fukuho was highly
186 susceptible to leaf rust and that the 3P substitution line I-15 was highly resistant to leaf rust (Fig. 4c).
187 However, the 3P addition line I-27 was susceptible to leaf rust(Fig. 4c). Different resistance to leaf rust
188 for the two materials indicated that the source of the 3P chromosome in the 3P addition line and
189 substitution line may not be the same chromosome. These results may be useful for improvements in
190 wheat disease resistance.

191 **Table 2** Evaluation of the agronomic traits of three materials in the 2019-2020 and 2020-2021 growing
192 seasons

Year	Materials	PH(cm)	FTN	SL(cm)	SN	SNPS	GNPS
2019-20	Fu	91.30±4.34	14.2±3.10	9.87±0.58	18.47±0.74	4.27±0.46	57.93±7.21
	I-27	112.52±6.25**	17.70±6.02*	11.11±1.07**	18.52±1.42	3.79±0.42	51.64±5.66
	I-15	134.71±7.26**	13.19±4.14	11.45±1.08**	20.43±1.63*	3.68±0.47	46.68±7.00*
2020-21	Fu	90.53±5.80	10.11±3.01	9.77±0.54	19.94±1.35	4.06±0.24	58.33±7.30
	I-27	116.33±2.45**	13.00±2.00**	10.61±0.89**	20.67±2.40	4.10±0.33	54.78±7.68
	I-15	142.44±3.64**	11.11±1.69	12.79±1.58**	24.33±2.24**	3.80±0.44	58.67±7.53

193 PH: plant height, FTN: fertile tiller number, SL: spike length, SN: spikelet number, SNPS: Spikelet number per
194 spike, GNPS: grain number per spike. The symbols "*" and "**" denote that the plants are significantly different
195 according to t tests at the probability levels of $P < 0.05$ and $P < 0.01$, respectively (t test performed by SPSS 25.0).

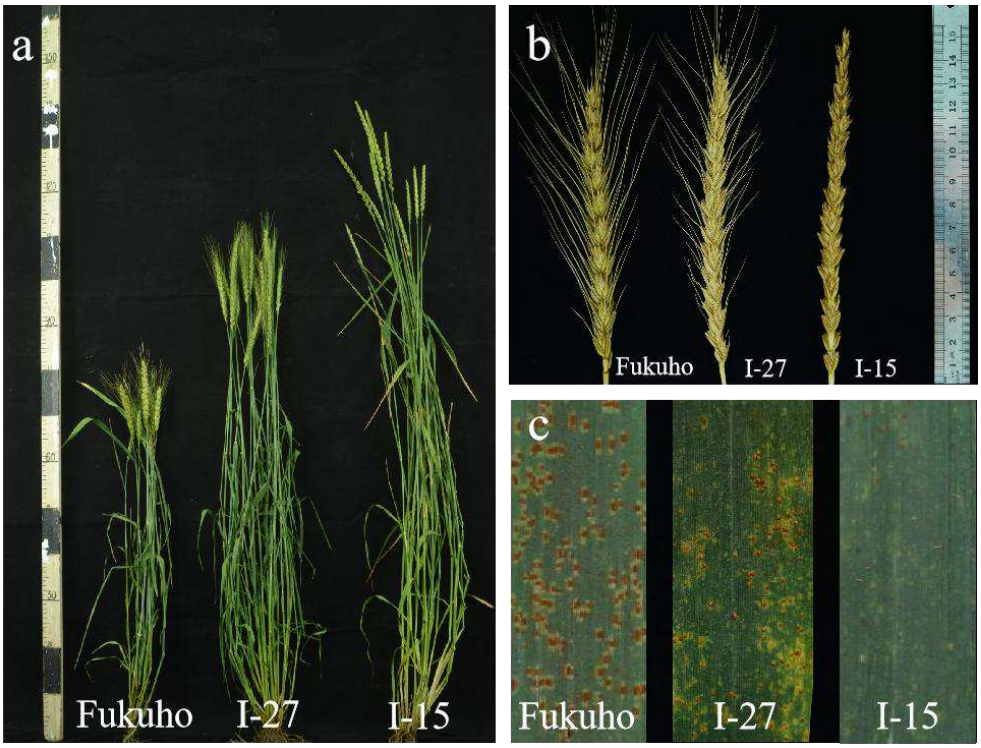

Fig. 4 Morphological traits of wheat–*A. cristatum* 3P addition line I-27, 3P substitution line I-15 and Fukuho. The whole plants(a), spike length(b) and disease response to leaf rust(c).

Discussion

Wild relatives of wheat carry a large number of genes that control excellent traits, and thus, these relatives represent a gene bank for wheat genetic improvement. Based on the partial homology between the genomes of wild relatives of wheat and that of wheat, we can genetically improve wheat by partial homoeologous recombination (Rey et al. 2018). Alien addition lines and substitution lines produced by distant hybridization and chromosome engineering are important intermediate materials for transferring exogenous excellent genes (Pan et al. 2017; Li et al. 2020; Song et al. 2020). In this study, a wheat–*A. cristatum* 3P disomic addition line and a wheat–*A. cristatum* 3P(3B) disomic substitution line were identified via molecular marker, GISH and FISH analysis, and these lines were relatively genetically stable. It has been demonstrated that the *A. cristatum* 3P chromosome carries genes that govern superior agronomic traits, such as multiple tillers and spikelets, increased spike length, and resistance to leaf rust. However, we found that although the spikelet number increased, the grain number per spike did not increase significantly, suggesting that the whole introduced chromosome may carry some genes with adverse effects. Therefore, we need to reduce the amount of exogenous fragments by creating small-fragment translocation lines to reduce the genetic burden, laying a foundation for the subsequent mapping of excellent gene of the *A. cristatum* 3P chromosome.

As an important wild relative of wheat, *A. cristatum* can provide many excellent traits and genes that have considerable potential for improving common wheat (Li et al. 1997). By using different wheat–*A. cristatum* addition lines, researchers have transferred many useful traits to common wheat, including resistance to biotic and abiotic stresses. Resistance to leaf rust and powdery mildew was

successfully conferred to wheat by transferring chromatin of *A. cristatum* chromosomes to common wheat (Jiang et al.2018; Li et al. 2020). Additionally, *A. cristatum* yield traits such as large spikes, multiple florets, multiple spikelets and high thousand-grain weight contained have also been successfully transferred to common wheat (Ye et al. 2015; Qi et al. 2021; Sun et al. 2021). The generation of the wheat–*A. cristatum* 3P addition line has resulted in the formation of a complete set of genetic materials for *A. cristatum* chromosome 1-7P addition lines. The complete set of 1-7P addition lines is a highly valuable tool for identifying excellent genes in the P genome and for studying the genetic evolutionary relationship between the P genome and wheat ABD genome.

Wheat leaf rust, which is caused by *Puccinia triticina* (Pt), has strong adaptability and is widely distributed (Yuan et al. 2020), is the main disease of wheat (Kolodziej et al. 2021) and affects wheat production in China and throughout the world (Savary et al. 2019). At present, breeding new resistant wheat varieties is still the most economical and effective control strategy for leaf rust. Seventy-nine wheat leaf rust resistance genes (*Lr1-Lr79*) have been designated, of which *Lr63* and *Lr66* have been mapped to chromosome 3A (Kolmer et al. 2010; Marais et al. 2010); *Lr27*, *Lr77* and *Lr79* are located on chromosome 3B (Singh et al. 1984; Kolmer et al. 2018; Qureshi et al. 2018), and *Lr22b* and *Lr69* have been mapped to chromosome 3D (Bartos et al. 1969; Qin et al. 2015). The above mentioned resistance genes were all from the homoeologous group 3 chromosome, while the resistance genes on the 3P chromosome of *A. cristatum*, a wild relative of common wheat, have not been reported. Unfortunately, our understanding of the resistance mechanisms of wheat against leaf rust is still limited. In addition, many researchers have found that leaf rust antigens are relatively scarce in the main wheat cultivars, while leaf rust races show continuous variation, resulting in the gradual loss of resistance in many resistant cultivars (Kokhmetova et al. 2021). Therefore, the identification of novel resistance genes will be useful for wheat leaf rust resistance breeding and analyzing the mechanisms underlying disease resistance.

Spikelet number is an important factor affecting wheat yield and is essentially a quantitative trait determined by many quantitative trait loci (QTLs) or genes. At present, a series of genes and QTLs responsible for multiple spikelets have been mapped to different chromosomes, such as 2A, 2B, 2D (Du et al. 2021), 3D (Li et al. 2021), 6BL (Katz et al., 2022), 5B, 7B (Tang et al. 2014a), 7A (Kuzay et al. 2019; Chen et al. 2020), 7DS (Chen et al. 2022), 1R (Yang et al. 2016), 2R (Dobrovolskaya et al. 2009), 2NS and 3NS (Zhao et al. 2019). However, the genes/QTLs controlling spikelet number on chromosome 3B have not been found. Thus, it is speculated that the multiple spikelets trait of the wheat–*A. cristatum* 3P(3B) substitution line is related to the *A. cristatum* 3P chromosome. The characteristics of multiple spikelets traits governed by genes carried by the *A. cristatum* 3P chromosome need to be evaluated further in the form of small-fragment translocation lines obtained by irradiation, which has potential application value in wheat breeding.

Growing evidence has demonstrated that homoeologous group 3 chromosomes of wheat carry many genes of excellent traits and genes that can be used for genetic improvement of wheat. Strong stripe rust resistance genes have been found in the wheat–*D. villosum* 3V(3D) substitution line (Zhang

et al. 2018). Kang et al. (2011) found that introgression of chromosome 3Ns from *Psathyrostachys huashanica* into wheat could also provide resistance to stripe rust. Guo et al. (2011) demonstrated that the *T. ponticum* 3E chromosome carries stripe rust resistance genes and showed excellent salt tolerance; The wheat–*Aegilops biuncialis* 3M addition line presented enhanced salt tolerance because of its ability to regulate the osmoprotection mechanism to increase sugar and proline accumulation (Darko et al., 2020). The wheat–*L. mollis* 3Ns#1(3D) substitution line carries leaf rust resistance gene(s), and its spike length and fertile tiller number are substantially improved (Pang et al. 2014). In addition, the FHB resistance gene *Fhb1* was mapped to wheat chromosome 3B (Su et al. 2019). In this study, it was found that wheat–*A. cristatum* 3P(3B) substitution line was highly resistant to leaf rust, and *A. cristatum* chromosome 3P could improve the spike traits of common wheat, indicating that there was certain differentiation and partial homology in homoeologous group 3 during the genetic evolution of wheat. Therefore, further exploration of the excellent genes of homoeologous group 3 of *A. cristatum* via generation of wheat–*A. cristatum* 3P addition and substitution lines is important.

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Statements and Declarations

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Conflict of interest: The authors declare no conflict of interest.

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