Uncovering macrosyntenic relationships between tetraploid *Agropyron cristatum* and bread wheat genomes using COS markers

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AUTHOR CONTRIBUTION STATEMENT
AC, JD, JV, IM and MS conceived the project. ACP, EG, JV, IM and MS performed the experiments and drafted the manuscript; all authors contributed to the manuscript writing and approved the final version.

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KEY MESSAGE
Using COS markers, the study reveals macrosyntenic relationships between tetraploid *Agropyron cristatum* and bread wheat to support alien introgression breeding of wheat.

ABSTRACT

Crested wheatgrass (*Agropyron cristatum* L. Gaertn.) is a wild relative of wheat that possesses many genes that are potentially useful in wheat improvement. The species comprises a complex of diploid, tetraploid and hexaploid forms. In this study, wheat-*A. cristatum* chromosome, telosome and translocation lines were used to characterize syntenic relationships between tetraploid *A. cristatum* and bread wheat. Prior to mapping COS markers, the cytogenetic stock lines were characterized for fertility and by FISH and GISH for karyotype stability. Out of 328 COS markers selected for the study, 279 consistently amplified products in tetraploid *A. cristatum*, and, out of these, 139 were polymorphic between tetraploid crested wheatgrass and wheat. Sixty-nine markers were found to be suitable for the detection of tetraploid *A. cristatum* chromosomes 1P - 6P in wheat, ranging from 6 to 17 markers per chromosome. BLASTn of the source ESTs resulted in significant hits for 67 ESTs on the wheat pseudomolecules. Generally, COS markers of the same homoeologous group were detected on similar arms in both *Agropyron* and wheat. However, some intragenomic duplications and chromosome rearrangements were detected in tetraploid *A. cristatum*. These results provide new insights into the structure and evolution of the tetraploid *A. cristatum* genome and will facilitate the exploitation of the wild species for introgression breeding of bread wheat.

Keywords: *Agropyron cristatum*, Bread wheat, Chromosome rearrangements, COS markers, Fluorescence in situ hybridization, Homoeologous relationships

INTRODUCTION

The gene pool of bread wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD) was narrowed down during thousands of years of domestication, cultivation and breeding. The dwindled genetic diversity hampers the development of cultivars with improved quality and tolerance to biotic and abiotic stresses. Furthermore, wild crop relatives were not subjected to human selection, they exhibit large genetic variation, and they represent an attractive source of alleles and genes for crop improvement (Tanksley and McCouch 1997). Interspecific hybridization is a promising tool to utilize the extant genetic diversity in wheat improvement through the chromosome-mediated transfer of useful agronomic traits (Feuillet et al. 2008).

The genus *Agropyron* includes 10-15 species (Asay and Jensen 1996; Martín et al. 1999; Liu et al. 2010) and is an attractive source of genes for wheat improvement (Asay and Johnson 1990; Limin and Fowler 1990; Dong et al. 1992; Friebe et al. 1992). The most widespread species of the genus is *A. cristatum* (L.) Gaertn (Yang et al. 2014), also known as crested wheatgrass, which is distributed in Eurasia and comprises a series of diploid (2n = 2x = 14), tetraploid (2n = 4x = 28), and hexaploid (2n = 6x = 42) forms (Löve 1982, 1984; Dewey 1984; Li et al. 2007). The diploids are less common and distributed from Europe to Mongolia, whereas tetraploids are widespread, particularly in central Europe, the Middle East, and central Asia. Hexaploids are rare and are mainly found in Turkey, Iran and Georgia (Copete et al. 2018; Dewey and Asay 1982)

To date, the effect of the lower crossability of diploid *A. cristatum* due to the disequilibrium in the endosperm balance number in wheat hybrid seeds (Chen et al. 1989) has not been solved, while hybridization between wheat and tetraploid *A. cristatum* can be easily done (Chen et al. 1989; Martín et al. 1999). Thus, out of the three ploidy levels, tetraploid crested wheatgrass is the most used form for hybridization with wheat (Chen et al. 1989, 1994; Li et al. 1997, 1998, 2016; Han et al. 2014; Ochoa et al. 2015; Soliman et al. 2007). Unfortunately, the nature of the tetraploid form remains obscure. According to Martín et al. (1999), tetraploid *A. cristatum* is not autopolyploid, and its two P subgenomes exhibit segmental autosomy (Stebbins 1947) that are distinguished from each other by structural rearrangements (Hsiao et al. 1989). Schulz-Schaeffer et al. (1963) also proposed the segmental allopolyploid nature of tetraploid and hexaploid *A. cristatum*, and Han et al. (2014) suggested that tetraploid *A. cristatum* originated from derivatives of hybridization between diploid *A. cristatum* and diploid *A. mongolicum.* Both species contain the same basic P genome, however, the genomes differ by structural rearrangements (Hsiao et al. 1989; Wu et al. 2006). In line with this, Zhao et al. (2017) suggested designating the P genomes of *A. cristatum* and *A. mongolicum* as P^c and P^m, respectively. In contrast, other authors consider tetraploid *A. cristatum* to be autopolyploid, originating from the diploid *A. cristatum* (Taylor and McCoy 1973; Vogel et al. 1999; Zhao et al. 2017). Although the nature of the tetraploid genome of *A. cristatum* and the origin of its P genome(s) have not yet been clarified, the tetraploid form is generally considered autotetraploid when used in development of wheat-*A. cristatum* introgressions. For all wheat-*A. cristatum* addition and translocation lines produced so far, only one of the two homologous pairs was introgressed into wheat (Chen et al. 1989, 1994; Li et al. 1997, 1998, 2016; Han et al. 2014; Ochoa et al. 2015).

Crested wheatgrass is a perennial species of economic importance as forage; it is facultatively allogamic, autocompatible, and shows high crossability with wheat and other Triticeae (Martín et al. 1998, 1999). A number of genes controlling traits of agronomic interest were identified in *A. cristatum*, including genes underlying resistance to barley yellow dwarf virus (Sharma et al. 1984; Shukle et al. 1987), wheat streak mosaic virus (Sharma et al. 1984; Brettell et al. 1988; Triebe et al. 1991), yellow rust, leaf rust and stem rust ~~and stripe rust~~ (Knott 1964, 1968; Cauderon and Rhind 1976; Whelan 1988; Friebe et al. 1992; Zhang et al. 2017), powdery mildew (Copete and Cabrera, 2017), cold tolerance (Limin and Fowler 1987), salinity tolerance (Dewey 1960, 1962; McGuire and Dvořák 1981; Forster et al. 1987; Littlejohn 1988), drought tolerance (Dewey 1984) and genes affecting yield (Song et al. 2013).

To date, no systematic attempts have been made to utilize these genes in wheat breeding. Nevertheless, there has been a growing interest in using *A. cristatum*, particularly the tetraploid form, in wheat improvement, and several research groups identified its chromosome 6P as the carrier of genes that control the number of florets and kernels per spike (Luan et al. 2010) in addition to a locus conferring stripe rust resistance (Zhang et al. 2017). Moreover, production of wheat-chromosome *A. cristatum* translocation lines was reported by Luan et al. (2010), Song et al. (2013) and Ochoa et al. (2015). Ochoa et al. (2015) developed translocation line TH4, which carries a compensating Robertsonian translocation involving the long arm of wheat chromosome 1B and the short arm of an unidentified tetraploid *A. cristatum* chromosome. The chromosome fragment transferred to bread wheat contributed a substantial level of partial resistance to leaf rust (Ochoa et al. 2015). The translocation line makes the disease resistance and other genes from tetraploid *A. cristatum* accessible for wheat breeding programs and indicates the feasibility of this approach.

Efficient introgression of genes from wild relatives *via* interspecific and intergeneric hybridization is facilitated by the knowledge of their genome structure. If collinearity between the donor and recipient genomes is broken down due to chromosome rearrangements, gene transfer by chromosome recombination may result in progenies with nonbalanced genomes (Devos et al. 1993; Zhang et al. 1998). Altered structure of the donor chromosomes may interfere with meiotic recombination and hamper attempts to reduce the size of introgressed chromatin to eliminate undesirable traits (Nasuda et al. 1998). An investigation of chromosome structure and cross-genome homoeology in diploid *A. cristatum* revealed evolutionary chromosomal reorganizations (Said et al. 2018). Chromosome rearrangements breakdown the collinearity between the homoeologous wheat and alien chromosomes. As a consequence, the genes on alien chromosome segments do not compensate for the loss of wheat genes and thus may negatively affect the agricultural performance of wheat-alien translocations.

It is known that polyploidization may induce genome rearrangements (Ma et al. 2004; Han et al. 2005, 2017; Zhang et al. 2013), and thus one may expect structural differences between the P genomes of diploid and tetraploid *A. cristatum*. This possibility was confirmed by Han et al. (2014), who found that tetraploid *A. cristatum* chromosome 6P differs from its wheat homoeologs by large rearrangements. This observation underlines the need for detailed analysis of the structure of all chromosomes of tetraploid *A. cristatum*. To date, knowledge of the tetraploid *A. cristatum* genome remains poor and, to date, cross-genome homoeology of tetraploid *A. cristatum* and wheat has not been analyzed in detail.

Efficient alien gene transfer requires appropriate methods for screening and characterization of interspecific hybrids, backcross progenies and alien introgression lines. Currently, the main tools for their selection and characterization are laborious cytogenetic methods, such as C-banding (Friebe et al. 1996), fluorescence in situ hybridization (FISH) (Rayburn and Gill 1985; Schwarzacher and Heslop-Harrison 2000; Schneider et al. 2005) and genomic in situ hybridization (GISH) (Schwarzacher et al. 1989; Le et al. 1989). However, the potential of FISH to identify particular chromosomes and their segments is compromised by the lack of suitable probes, and the cytogenetic methods suffer from low sensitivity to detect small introgressed segments (Choi et al. 2009).

The availability of molecular markers capable of detecting small segments of *A. cristatum* chromatin introduced to wheat would be very useful for marker-assisted alien introgression breeding of wheat (Copete and Cabrera 2017). However, the efficiency of introgression breeding and development of high-density genetic maps for *A. cristatum* has been limited by the small number of molecular markers available for high-throughput screening (Han et al. 2014). Progress in this area has been slow for *A. cristatum,* and specific markers have been developed only for chromosomes 6P (Cheng et al. 2012) and 7P (Lu et al. 2016) from tetraploid *A. cristatum*. Development of DNA markers is greatly facilitated by the availability of genome

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Comentado [Dori2]: Friebe et al 1991

Comentado [Dori3]: Stripe rust = yellow rust

sequences. However, production of genome sequence assemblies is still not trivial in species with large and complex genomes such as *A. cristatum* with a 1C genome size of 6,352 Mbp (Said et al. 2018). This fact underlines the significance of exploiting molecular markers from genetically related species and their assignment to individual chromosomes of the donor genome using alien introgression and/or translocation lines (Said and Cabrera 2009; Cherif-Mouaki et al. 2011; Said et al. 2012; Copete and Cabrera 2017). While aneuploids are attractive material for assignment of markers to alien chromosomes, one has to be aware of the fact that chromosome composition of lines carrying alien chromatin may change in subsequent generations and that the introgressed chromatin may be lost (Szakács and Molnár-Láng 2010). This highlights the importance of validating the long-term karyotype stability of introgession lines used for molecular marker analyses.

Alien addition and translocation lines are an ideal template for PCR-based mapping to assign molecular markers to chromosomes of the wild relatives of wheat (Said and Cabrera 2009; Cherif-Mouaki et al. 2011; Said et al. 2012; Han et al. 2014; Ochoa et al. 2015; Copete and Cabrera 2017; Zhang et al. 2017; Ma et al. 2018). Using gene-based conserved orthologous set (COS) markers on wheat-*Aegilops* introgression lines, Molnár et al. (2013, 2016) assigned a total of 100 markers on 544 loci to the U, M, S and C genome chromosomes of *Aegilops* spp. The genomic position of ortholog unigene EST-contigs used for the COS marker design made it possible to investigate macrosyntenic relationships between *Aegilops* and wheat using *Brachypodium* and rice as a reference (Molnár et al. 2016). In this work, we used genomic DNA from thirteen wheat-*A. cristatum* chromosome and telosome addition lines and a translocation line carrying the short arm of chromosome 1P for PCR with COS markers to obtain new insights into the macrosyntenic relationships at the chromosome level between the genomes of tetraploid *A. cristatum* and bread wheat.

MATERIALS AND METHODS

Plant material

The seeds of diploid *A. cristatum* cv. Parkway (2n = 2x = 14, PP), accession number PI 415799 were provided by Dr Joseph Robins (ARS Forage and Range Research Laboratory, USDA, Logan, USA). The seeds of tetraploid *A. cristatum* (2n = 4x = 28, PPPP) accession PI 222957 were obtained from the USDA genebank (http://www.ars-grin.gov). The seeds of bread wheat cv. Chinese Spring (CS), as well as the seeds of wheat-*A. cristatum* chromosome addition lines (CS-P) carrying chromosomes 1P, 2P, 3P, 4P, 5P and 6P (CS-1P-6P), telosome addition lines (CS-PS, PL) for chromosome arms 2PS, 2PL, 4PS, 5PL, 6PS and 6PL, and a wheat-*A. cristatum* translocation line (CST-P) carrying a compensating Robertsonian translocation involving the long arm of bread wheat CS chromosome 1B and the short arm of an unidentified chromosome from tetraploid *A. cristatum* (Ochoa et al. 2015), were provided by Dr Adoración Cabrera (Genetics Department, University of Córdoba, Spain). The production of the new line CS-3PS in the present study was carried out at IEB by successive selfing line CS-3P possessing one 3P chromosome and one P telosome and selecting for the genotypes carrying mono- or ditelosomic 3PS (Fig 1).

Long-term stability of the karyotype, fertility and morphological characteristics

Before the COS marker analysis, all wheat-*A. cristatum* chromosome and telosome addition lines and the translocation line were characterized for fertility and karyotype stability during three successive generations. Fertility and spike morphological characteristics were estimated from five spikes for the following traits: spikelets per spike, seeds per spike and spike length. An analysis of variance was carried out and a mean values comparison was performed using the least significant difference method ($P \leq 0.05$). Statistical analysis was performed with Minitab 18 (www.minitab.com). Karyotype stability of the wheat-*A. cristatum* lines was evaluated by chromosome counting and genomic in situ hybridization (GISH) as detailed in Szakács and Molnár-Láng (2010). Chromosome compositions of the wheat-*A. cristatum* lines over the generations were expressed in percent (%): the number of plants with a specific chromosome composition divided by the total number of plants analyzed from a specific line and multiplied by 100.

Preparation of probes for FISH

A probe for *A. cristatum* tandem repeat ACRI_CL78 (Said et al. 2018) was labeled by PCR with digoxigenin-dUTP (Roche, Mannheim, Germany) using diploid *A. cristatum* cv. Parkway DNA as a template. Biotin-dUTP (Roche) or digoxigenin-dUTP labeled probe for 5S rDNA was prepared according to Fukui et al. (1994) using rice DNA as a template for PCR. The plasmid pTa71 (45S rDNA) containing a 9-kb fragment from bread wheat with 18S-5.8S-26S rDNA and intergenic spacers (Gerlach and Bedbrook 1979) and genomic DNA of tetraploid *A. cristatum* PI 222957 were labeled with either biotin or digoxigenin by nick-translation using standard kits (Nick Translation Mix, Roche) following the manufacturer's

instructions.

Mitotic chromosome preparation

Seeds were germinated on moistened filter paper in a glass Petri dish in the dark at 25 °C for 3-4 days. Root tips were transferred to distilled water and incubated overnight at 1 °C in a box filled with ice-water. Subsequently, the root tips were fixed in ice-cold 90% acetic acid for 10 min followed by three washes in 70% ethanol and stored in 70% ethanol at -20 °C. Chromosome preparations were prepared using the drop technique according to Kato et al. (2004, 2006), with minor modifications as described in Danilova et al. (2012).

Fluorescence in situ hybridization

Labeled probes for FISH and GISH were localized following the protocols of Cabrera et al. (2002) and Said et al. (2018) with modifications. Briefly, digoxigenin-labeled probes were detected using anti-digoxigenin fluorescein isothiocyanate (Roche). Biotin-labeled probes were detected with Cy3-conjugated streptavidin (Invitrogen, Life Technologies, Carlsbad, USA). The hybridization mixture (total volume = 10 µl/slide) contained 50 ng labeled probe DNA, 50% v/v formamide, 2 × SSC (0.15 mol/l NaCl plus 0.015 mol/l sodium citrate), 10% w/v dextran sulphate, 0.4 µg salmon sperm DNA and 0.1% w/v sodium dodecyl sulphate. In the case of GISH, 5 µg wheat genomic DNA was included in the hybridization mix as blocking DNA. The chromosomes and probes were denatured together at 80 °C for 3 min under high moisture conditions. The hybridization was carried out overnight at 37 ºC. The slides were washed, the hybridization sites were detected, and chromosomes were mounted and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield media (Vector Laboratories, Burlingame, USA).

Microscopy, software, signal capture and image analysis

Chromosome preparations were examined using an Axio Imager Z.2 Zeiss microscope (Zeiss, Oberkochen, Germany) equipped with a Cool Cube 1 (Metasystems, Altlussheim, Germany) camera and appropriate optical filter sets. The signal capture and image processing were performed using ISIS software (Metasystems). The final image adjustment was done in Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, USA).

COS-marker analysis

Genomic DNA was extracted from young leaves of wheat-*A. cristatum* chromosome and chromosome arm addition and translocation lines, from the diploid and tetraploid *A. cristatum* accessions and from bread wheat cv. Chinese Spring, using a Quick Gene-Mini80 (FujiFilm, Tokyo, Japan) with a QuickGene DNA tissue kit (FujiFilm, Tokyo, Japan) according to the manufacturer's instructions and was used as a template for PCR. Primers for 328 COS markers covering wheat homoeologous groups I - VII (I: 76, II: 16, III: 23, IV: 120, V: 65, VI: 15 and VII: 13) were chosen from publicly available marker collections (Quraishi et al. 2009; Howard et al. 2011). Primer sequences for these markers and annealing temperature (Ta) are summarized in Supplementary Data S1. PCR was performed in 12 µl reaction volumes as described by Molnár et al. (2014, 2016) using a touchdown reaction profile: 94 °C 2 min, 10 cycles of 94 °C 0.5 min, Ta + 5 °C 0.5 min and decreased by 0.5 °C increments for every subsequent set of cycles, 72 °C 1 min, 30 cycles of 94 °C 0.5 min, Ta °C 0.5 min, 72 °C 1 min, hold at 72 °C 2 min in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). PCR products of the 112 markers, which were analyzed in MTA ATK MGI (Martonvásár), were separated by a Fragment Analyzer Automated CE System equipped with a 96-Capillary Array Cartridge with an effective length of 33 cm (Advanced Analytical Technologies, Ames, USA) and the results were analyzed and visualized by PROsize v2.0 (Advanced Analytical Technologies). The products of the remaining 216 markers, which were analyzed at the University of Córdoba, were separated on 2.5% agarose gels along with the O'RangeRuler™ 50 bp DNA size marker (Fermentas, Vilnius, Lithuania) as described by Nagy et al. (2006). The patterns were documented and analyzed using a GeneGenius gel documentation system (Syngene, Cambridge, UK).

Sequence analysis

To compare orthologous regions between the A, B or D genomes of bread wheat and the P genome of tetraploid *A. cristatum* identified by COS markers, a physical map was constructed for each of the wheat chromosomes showing the position of the source EST of the COS markers assigned to tetraploid *Agropyron* chromosomes. To do this, the EST source sequences (Supplementary Data S2) were used as queries in BLASTn searches against the wheat reference pseudomolecules (Consortium (IWGSC) et al. 2018) to identify the start positions (bp) of the ESTs. Throughout the study, BLAST hits with E-values smaller than

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2.8e^{-08}, Identity % > 58.44 and Alignment length > 100 bp were considered significant. The genomic start positions in bp of the best hits in wheat pseudomolecules (Supplementary Data S3) were used to construct physical maps of the polymorphic COS markers. The centromere positions for each wheat chromosome were determined from the wheat reference genome sequence (Consortium (IWGSC) et al. 2018). The length in bp of wheat pseudomolecules, as well as the start genomic positions of the ESTs, were converted to pixels and the physical maps of the COS markers were designed using custom-made software.

ACCESSION NUMBERS:

Plant material; diploid *A. cristatum* cv. Parkway (2n = 2x = 14, PP), accession number PI 415799 and tetraploid *A. cristatum* (2n = 4x = 28, PPPP) accession PI 222957 are available at the USDA genebank (http://www.ars-grin.gov). Wheat-*A. cristatum* chromosome additions (1P, 2P, 3P, 4P, 5P and 6P) and telosome additions (2PS, 2PL, 3PS, 4PS, 5PL, 6PS and 6PL) as well as a wheat-*A. cristatum* 1PS·1BL translocation line are available upon request: Mahmoud Said (said@ueb.cas.cz), Institute of Experimental Botany, Šlechtitelů 31, CZ-78371 Olomouc, Czech Republic.

RESULTS:

Karyotype stability of the wheat-*A. cristatum* chromosome addition and translocation lines

Karyotype stability was observed in a majority of the addition and translocation lines (Supplementary Table S1). Complete stability was observed in CS-6PL addition- and CST-P translocation- lines with 100% maintenance of the disomic state of the alien chromatin. The second greatest stability was observed for addition lines CS-1P, CS-4P, CS-5P, CS-6P, CS-5PL and CS-6PS, where more than 90% of the progeny retained the disomic state. The CS-2P, CS-2PL, CS-3PS and CS-4PS addition lines were relatively stable, as more than 77% disomic progeny plants were identified. Furthermore, the CS-2PS addition line was unstable, with 47% ditelosomic and 10% monotelosomic progeny, while the remaining 43% plants did not retain any alien chromatin. Line CS-3P was the most unstable, as a high proportion of plants (74%) did not retain any alien chromatin, 17% of the progenies were monotelosomic and 9% of the plants retained one chromosome 3P in addition to a chromosome arm later identified by FISH as 3PS. The 3PS chromosome arm was also detected in a new ditelosomic addition line CS-3PS, which was generated during the course of the study. This line showed high stability, where 77% of progenies maintained the ditelosomic state. Although the CS-6PS addition line was considered highly stable with 90% ditelosomic plants in the progeny, 5% of plants carried isochromosome 6PS and the remaining 5% were monotelosomic as revealed by GISH. The wheat-*A. cristatum* lines with disomic and ditelosomic additions confirmed by GISH (Fig 2, Supplementary Fig S1) were selected for COS marker analyses.

We observed two karyotypes in the progenies of line CS-3P, one retaining one chromosome 3P and one arm 3P, and the other karyotype of a new line possessing two telosomes (Fig 1). Based on the FISH pattern of the tandem repeat ACRI_CL78 (Said et al. 2018), the telosomes were identified as a homologous pair 3PS of a new ditelosomic addition line CS-3PS (Supplementary Fig S2). Furthermore, the telosomes of the new line CS-3PS had similar FISH pattern to the short arm of chromosome 3P of line CS-3P (Supplementary Fig S2). The results were also confirmed by the comparison of the FISH pattern of the tandem repeat on the short arm of chromosome 3P and the telosome in line CS-3P possessing one chromosome 3P and one arm (Supplementary Fig S2). The unidentified tetraploid *A. cristatum* chromosome short arm translocated to wheat chromosome arm 1BL (Ochoa et al. 2015) in the translocation line CST-P was identified in the present work by FISH as 1PS based on the molecular karyotype of *A. cristatum* (Said et al. 2018), as it is possible to distinguish between chromosomes 1P and 5P. Although both are characterized by 45S rDNA signals at the terminal position of the short arms, chromosome 5P has a 5S rDNA locus at the subterminal position of the short arm. This was also confirmed by comparing the distribution patterns of 45S rDNA on this chromosome arm in the translocation line with the patterns of 5S and 45S rDNA on tetraploid *A. cristatum* PI 222957 (Supplementary Fig S3), which was used by Ochoa et al. (2015) to develop the translocation. Consequently, based on these observations, the translocation line (CST-P) was renamed to CST-1PS·1BL.

The new wheat-*A. cristatum* CS-3PS ditelosomic line and the translocation line CST-1PS·1BL were also involved in the COS marker study (Fig 2, Supplementary Fig S1). Because the whole 3P chromosome was not represented in the set of wheat-*A. cristatum* disomic addition lines, we used line CS-3P which possesses one chromosome 3P and one telosome 3PS (Supplementary Fig S1).

Fertility and morphological traits

The observations on seed fertility and spike morphology are summarized in Supplementary Table S2 and Fig 3. All lines were fertile and vigorous over the generations, both in a greenhouse and under field conditions. The lines differed in spike morphology in terms of color, size and shape, and statistically significant differences were found between the lines for the evaluated characters. In particular, translocation line CST-1PS·1BL had a significantly longer spike length compared to CS, while the CS-1P addition line had the shortest spike with approximately half the spike length of CS. Line CS-1P had significantly fewer spikelets per spike compared to the remaining lines, while CS-2PL showed the significantly highest value for this trait. With respect to seed number per spike, CS-6P had a greater mean value, but the difference as not significant when compared to CS, CS-4PS and CS-6PL. Line CS-2P had the lowest number of seeds per spike, but the difference was not significant compared to CS-1P and CS-4P. In this study, all wheat-*A. cristatum* lines yielded awnless spikes, except for line CS-2P which had awned spikes, and fewer and shorter awns were also observed on the upper spikelets of CS-2PS and CS-2PL (Fig 3).

Assignment of COS markers to P chromosomes

The confirmation of the presence of chromatin originating from tetraploid *A. cristatum* in wheat-*A. cristatum* addition and translocation lines made them suitable for the subsequent COS markers analysis. Out of the 328 markers tested for transferability to tetraploid *A. cristatum* (Table 1), 279 (85.1%) consistently amplified products in tetraploid *A. cristatum* PI 222957, and, out of these, 139 (49.8%) were polymorphic between tetraploid *A. cristatum* and wheat (CS) (Fig 4 and Table 1). The highest level of polymorphism (90.0 - 90.9%) was observed for the wheat chromosome group III and VI markers, while the wheat chromosome group I and IV markers showed a relatively low level of polymorphism (32.0-35.0%). Using wheat-*A. cristatum* disomic- and ditelosomic addition lines, sixty nine out of the 139 polymorphic markers were assigned to the P-genome chromosomes. Because some markers were assigned to more than one P chromosome (Table 2), the total number of markers (69) assigned to tetraploid *A. cristatum* chromosomes was different from the sum (78) of the specific markers per P chromosome (No. of markers/No. of PCR amplicons per chromosome: 1P: 11/15; 2P: 6/7; 3P: 10/14; 4P: 19/24; 5P: 21/24; 6P: 11/13).

The availability of CS-*A. cristatum* ditelosomic lines provided the opportunity to locate COS markers on chromosome arms. Because ditelosomic lines for the short and long arms were available only for chromosomes 2P and 6P, only one of the two arms could be checked for chromosomes 3P, 4P and 5P and for 1P using the CST-1PS·1BL translocation. Therefore, if the PCR results were negative in the available telosomic line and positive in the whole chromosome addition line, we concluded that the COS marker was located on the opposite arm. In this manner, sixty-seven out of the sixty-nine markers were assigned to chromosome arms of the tetraploid *A. cristatum* (Table 2). We failed to map the remaining two markers to particular chromosome arms. The tetraploid *Agropyron* chromosome-specific markers showed a significant level of length polymorphism (3 - 558 bp, mean: 54.59 bp) between wheat and the parental tetraploid *A. cristatum* genotype represented by the wheat-*A. cristatum* addition and ditelosomic lines. Therefore, they were considered suitable for marker-assisted selection of new wheat-*Agropyron* introgression lines in prebreeding programs. In this study, 90 polymorphic loci of 69 markers (1-3 loci, 1.30 loci per marker) covering from one to six homoeologous groups of the P genome were found to be suitable for high-throughput detection of tetraploid *A. cristatum* chromosomes.

Wheat-*A. cristatum* homoeology at the chromosome level

To investigate wheat-*A. cristatum* macrosyntenic relationships at the chromosome level, the source ESTs of the 69 polymorphic COS markers were BLASTed to the sequences of the wheat chromosomes (Consortium (IWGSC) et al. 2018). Sixty-seven ESTs marker showed hits on wheat pseudomolecules, and two markers (TR451, TR430) gave no hits. Seven markers (TR37, TR85, c750766, c756425, c746156, c759134 and TR764) were excluded from the subsequent analysis because the alignment length was below the threshold (100 bp). For the remaining markers, the start positions of the alignments of the best hits on the A, B and D genomes were extracted to produce a physical map from the perspective of the wheat genome (Supplementary data S3). In the map, the chromosomal locations of the markers in tetraploid *A. cristatum* were visualized by different colors of the marker names, which provides an overview of the wheat-*A. cristatum* genome relationships from the perspective of the wheat genome (Fig 5). In the physical map, the coverage of wheat chromosomes groups II, III and VI with COS markers was smaller (6-7 markers per chromosomes group) than wheat chromosome group I (10 markers) and wheat chromosome groups IV and V (15-16 markers per chromosome).

Most of the markers (48) were located on the same homoeologous (H) chromosome group in tetraploid *Agropyron* as in wheat, seven markers were assigned to nonhomoeologous P-genome chromosomes, while the remaining five markers were located on homoeologous and nonhomoeologous (HN) tetraploid *A. cristatum* chromosomes. The marker c757404, which is located on the interstitial part of the short arm of wheat chromosome group I, was assigned to the long arm of 5P. The marker TR72, specific to the subtelomeric region of wheat chromosome group II short arms, was located at 3P. The marker TR4, specific to the subtelomeric region of short arms of wheat chromosome group III was assigned to 5PL, while TR390, specific to the long arms of the interstitial part of wheat chromosome group III was located at 1P. On wheat chromosome group IV, two markers (TR118 and TR133) were mapped to the 5P chromosome and one marker c797119 was assigned to 6P. The wheat chromosome group V markers TR390, TR759 and TR471 were detected on chromosomes 1P, 6P and 3P, respectively, while the duplicated locus TR636, which is located on wheat chromosome groups II and VI, was identified on 2P.

Chromosomal and subchromosomal synteny distortions in the P genome

As shown in Table 2 and in the physical map (Fig 5), 53 (48 (H) and 5 (HN)) out of 60 (88.3%) of the COS markers showed synteny between the bread wheat and tetraploid *A. cristatum* genomes as they were detected on the same homoeologous chromosome groups, with 46 (76.7%) of them at the same short or long arm in tetraploid *Agropyron* and wheat. However, the remaining seven (11.7%) COS markers mapped to nonhomoeologous chromosomes and thus revealed structural differences between the chromosomes. Ten markers (16.7%) showed another kind of chromosome alteration, where markers specific for the long arm of wheat chromosomes were found at the short arm of the same homeologous chromosomes in tetraploid *Agropyron* and vice versa. This kind of synteny perturbation between the homoeologous chromosome groups of wheat and tetraploid *A. cristatum* was found in group I chromosomes where two markers on the short arms of wheat chromosomes group I (c740349, c743346) were located on the long arm of 1P (Fig 5). In wheat chromosome group III, two markers in the pericentric region of long arms (c767527, TR63) were located on 3PS. In wheat chromosome group IV, the marker TR188 from 4AS and 4BL and marker TR113 were located on the short arms of 4B and 4D, and 4AL were both located on 5PL, while the marker c797119 from 4AS was detected on 6PL. The marker c756721, specific for the distal third of wheat chromosome group V, was found on 5PS, while the wheat chromosome group VI markers BE445667 and c724406, located at the pericentric region of the short and long arms, respectively, were detected on the opposite arm of 6P. Furthermore, the chromosomal location of other COS markers revealed some intragenomic duplications in tetraploid *A. cristatum* relative to wheat (Table 2 and Fig 5). Three duplications were detected by markers specific to wheat chromosome group II. Loci for marker *2R* on the short arms of wheat chromosome group II detected a duplication of 2PL/5PL, while on the long arms, two duplications 2PL/6PS and 2PL/5PL/6PS were found by the markers *c744070* and *2N*, respectively. One duplication, 1PL/3PL/4PL/6PL, was detected by the marker c803223, which is specific for the telomeric region of wheat chromosome group III, while a 4PL/6PS duplication was found by the marker BE426214, which is specific for the long arms of wheat chromosomes group VI.

Comentado [Dori5]: In table 2 the TR markers are in lower case

Comentado [Dori6]: Not fount in table 2

Comentado [Dori7]: The arm location in wheat do not appear in Table 2. In any case, the arm location of BE445667 agree in both wheat and 6P (in the short arm). Why is not the wheat arm location in table 2 of these markers?

Comentado [Dori8]: In italic?

DISCUSSION

The chromosomes of tetraploid *A. cristatum* were transferred to bread wheat CS by Chen et al. (1989). Subsequently, the identity of individual P chromosomes in the wheat-*A. cristatum* addition lines was confirmed by RFLP markers identifying each homoeologous chromosome arm (Chen et al. 1994). Since the tetraploid *A. cristatum* parental genotype used by Chen et al. (1989) was inaccessible to us, in the present work we used tetraploid *A. cristatum* accession PI 222957, which was used by Ochoa et al. (2015) to generate translocation line CST-1PS·1BL, also made in bread wheat CS. Due to the unavailability of chromosome addition line 7P, we were not able to assign COS markers to this chromosome. Disomic, monosomic, ditelosomic, telosomic, and plants carrying isochromosomes were identified in the progenies of wheat-*A. cristatum* lines (Supplementary Table S1).

Prior to investigating wheat-*A. cristatum* macrosyntenic relationships, wheat-*A. cristatum* chromosome addition, chromosome arm addition and translocation lines were evaluated for cytogenetic stability, fertility, and spike morphology. Our observations agree with Taketa et al. (1995), Molnár-Láng et al. (2005) and Szakács and Molnár-Láng (2010), who observed elimination of alien chromatin from a wheat host genome. Nevertheless, we identified a disomic state of alien chromosomes, telosomes or translocations in the progenies of each wheat-*A. cristatum* line, except for CS-3P. This observation is in line with other reports that described the difficulty of maintaining homoeologous chromosome 3 introduced from wild relatives in wheat (Miller et al. 1982; Said et al. 2012). Collectively, these results indicate that wheat

cytogenetic stocks carrying chromosome 3 from wild relatives of wheat are difficult to maintain in the disomic state. Chen et al. (1994) tested this line using molecular markers from wheat homoeologous chromosome arms of group III, but the authors were not able to distinguish between the monosomic and disomic sates. Our GISH analysis showed that CS-3P line carried one chromosome and one telosome from tetraploid *A. cristatum,* which segregated to a new ditelosomic addition line, where *A. cristatum* chromatin was identified by FISH as 3PS. Said et al. (2018) characterized chromosome arm 3PS by subterminal FISH signal from the tandem repeat ACRI_CL78, which is exactly what we found in the present work on the telocentric chromosome. Li et al. (1997, 1998, 2016) obtained addition lines for chromosome 1P-7P in the background of wheat cv. Fukuhokomugi. However, so far there are no reports of karyotype stability of these lines.

Ochoa et al. (2015) suggested that the chromosome arm in the wheat-*A. cristatum* translocation line CST-1PS·1BL that has been transferred to 1BL was the short arm of chromosome 1P. They also found that this fragment compensated for the lack of a 1BS arm. In tetraploid *A. cristatum* PI 222957, the source of *A. cristatum* chromatin for this translocation, we identified two pairs of chromosomes carrying 45S rDNA, where one of them also carried 5S rDNA. According to Said et al. (2018), the two chromosome groups were identified as 1P and 5P. In the present study, simultaneous localization of 5S rDNA and 45S rDNA by FISH in tetraploid *A. cristatum* and the wheat translocation line CST-1PS·1BL allowed for distinguishing between these chromosomes and clearly identifying the chromatin segment transferred from tetraploid *A. cristatum* to bread wheat as 1PS.

Our observations confirm that cytogenetic stocks require cytological examination to verify their stability. According to O'mara (1940), Riley and Chapman (1958) and Evans and Jenkins (1960) disomic wheat alien additions express diagnostic morphological traits such as plant stature, spike shapes or seed fertility. However, in the case of the wheat-*A. cristatum* addition and translocation lines used in the present study, the differences were not clearly manifested by all genotypes to a point that allowed us to identify their chromosome composition. For instance, the spike morphology of the CS lines carrying tetraploid *A. cristatum* chromatin in a disomic, monosomic, ditelosomic, telosomic or isochromosome state were similar to a large extent, showing predominantly CS characters. The only exceptions were the addition lines CS-1P, whose spikes are shorter and more square-headed, CS-2P, CS-2PS and CS-2PL which produced awned spikes, and the CST-1PS·1BL translocation line, whose spikes were the longest, the narrowest and had a dark color. Similar observations were made by Szakács and Molnár-Láng (2010) on the morphology of the spikes of wheat CS chromosome additions from other wild relatives. Cytogenetic characterization of these lines made it possible to picture the crested wheatgrass chromatin in the background of wheat and enabled their safe use for COS marker analysis.

Easy to use, chromosome-specific molecular markers are a prerequisite for increasing the selection throughput of wheat-alien introgression lines with desirable karyotypes. The present study significantly increased the number of PCR-based markers available for detection of chromosomes 1P - 6P of tetraploid *A. cristatum* and their arms in the wheat background. We observed 85.1% transferability of COS markers between wheat and tetraploid *A. cristatum*, which is less than reported by Linc et al. (2017), who investigated COS markers on diploid *A. cristatum* and found 92.1% transferability between wheat and *A. cristatum*, but is higher than those found by Copete and Cabrera (2017), who obtained 68.2% transferability of wheat chromosome group II and VI specific COS markers between wheat and tetraploid *A. cristatum*.

We found that out of the 279 COS markers producing amplicons, 139 (49.8%) were polymorphic between wheat and tetraploid *A. cristatum*. A similar range of size polymorphism (54.27%) was reported for EST-SSR markers between the wheat cultivar 'Fukuhokomugi' and tetraploid *A. cristatum* genotype Z559 by Han et al. (2014). Interestingly, the same work showed a much smaller percentage of size polymorphism (36.95%) for genomic SSR markers. Our work significantly augmented the number of P chromosome-specific markers by identifying the chromosomal locations of 69 COS markers covering the 1P-6P chromosomes from tetraploid *A. cristatum*. These polymorphic markers are considered potentially useful to follow tetraploid *A. cristatum* chromosomes in bread wheat backgrounds during prebreeding programs. Macrosyntenic relationships between wheat and related species provide important information for the targeted development of markers specific to alien chromosome regions potentially responsible for important agronomic traits as demonstrated for *Aegilops ventricosa* (Burt and Nicholson 2011).

The present study revealed close homeologous relationships between the chromosome arms of bread wheat and tetraploid *A. cristatum*. However, this arm-level homoeology was perturbed in some loci. On wheat chromosome group I, one marker on the short arm indicated partial homoeology with 4P, one marker on the wheat chromosome group II short arm reflected homoeology with 3P and others indicated 2P/5P or 2P/5P/6P duplications. Homoeologies to 5P and 1P were also detected on the short and long arms of wheat chromosome group III, respectively. We also detected wheat chromosome group IV loci related to 5P or 6P, wheat chromosome group V loci related to 1P, 3P or 6P, and a locus on the wheat chromosome group VI short arm that was related to 2P. Our results on the macrosyntenic relationships between wheat and tetraploid *A. cristatum* agree well with those of recent comparative genomics studies. Using the wheat 660k SNP array to genotype a diploid *A. cristatum* x *A. mongolicum* segregating population, Zhou et al. (2018) also found that the P genome of *Agropyron* is collinear and relatively conserved relative to wheat genomes. The authors also identified rearrangements and introgressions in the P genome relative to wheat. For each homoeologous group, most of the markers located on the short or long arms of wheat chromosomes group I - VI were assigned to the same short or long arms of 1P - 6P chromosomes, respectively. However, we observed the presence of some wheat short- or long-arm markers on the opposite arms in tetraploid *A. cristatum* in wheat chromosome groups I and III - VI. These intrachromosomal perturbations of wheat-*A. cristatum* macrosynteny might be related to extensive intrachromosomal rearrangements, such as peri- and paracentric inversions.

Using the single-gene FISH method to compare the chromosome structure of diploid *A. cristatum* with those of bread wheat, Said et al. (2018) found important structural rearrangements for chromosomes 2P, 4P, 5P, 6P and 7P. For instance, a pericentric chromosome inversion on 4P and a paracentric inversion on 6PL were observed. Furthermore, reciprocal translocations between 2PS and 4PL were discovered. Our results based on the chromosomal location of COS markers may indicate that chromosomal inversions are more abundant in the P genome of tetraploid *A. cristatum* than those of the diploid form. This is in agreement with previous studies suggesting that polyploidization induces genome reorganization (Ma et al. 2004; Han et al. 2005, 2017; Zhang et al. 2013). These findings demonstrate that evolutionary chromosomal rearrangements involving inversions occurred at the subchromosomal level either in the genome of wheat or tetraploid *Agropyron*. However, further high-resolution genome analyses of more accessions are needed to obtain more insight into the genome structure of tetraploid *A. cristatum*.

CONCLUSIONS

In the present study, a set of COS markers was successfully assigned to the chromosomes and chromosome arms of the P genome of tetraploid *A. cristatum*, which is the only form of this wild species suitable for chromosome-mediated gene transfer to bread wheat. Our results revealed the genome structure and the macrosyntenic relationships of this species relative to wheat, which could help us to understand the evolution of species from the Triticeae tribe, open the door for genome analysis and support the use of this important wild gene source in wheat breeding.

COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there are no conflicts of interest.

SUPPLEMENTARY DATA

Electronic supplementary material: The online version of this article contains supplementary material.

Online Resource 1 (File name: ESM_1) containing Figures:

Supplementary Fig. S1 GISH on mitotic metaphase plates in wheat-*A. cristatum* chromosome addition lines (a-f), ditelosomic addition lines (g-m) and homozygous translocation line 1PS·1BL (n) using genomic DNA from *A. cristatum* (green). The chromosomes were stained by DAPI (blue). Bars = 10 μm.

Supplementary Fig. S2 FISH and GISH on chromosomes 3P and 3PS in the genetic background of wheat. **a**) The FISH pattern of the probe for ACRI_CL78 repeat (green) on chromosome 3P (left) and 3PS (right), in wheat-*A. cristatum* addition line monosomic 3P and monotelosomic 3PS. **b**) The FISH pattern of probe ACRI_CL78 (green) on 3PS in the wheat-*A. cristatum* 3PS ditelosomic addition line. **c**) GISH using DNA from *A. cristatum* (red) and FISH with a probe for ACRI_CL78 (green) on chromosome 3P and 3PS in the

genetic background of wheat. **d**) GISH using DNA from *A. cristatum* (red) and FISH with a probe for ACRI_CL78 (green) on chromosome 3PS in the genetic background of wheat. *A. cristatum* chromatin is visualized by red color, whereas wheat chromosomes are counterstained with DAPI (**c** and **d**). The scale bar is 10 µm.

Supplementary Fig. S3 FISH and GISH for identification of the 1PS arm in the background of wheat. **a**) unknown *A. cristatum* chromosome short arm (arrows) translocated to wheat chromosome arm 1BL, and detected by GISH using DNA from *A. cristatum* (green) and 45S rDNA signals (red) by Ochoa et al. (2015). **b**) Probe for 45S rDNA (green) localized on the short arms of four pairs of chromosomes of the autotetraploid *A. cristatum* PI22297, which was used for the development of the translocation. Two pairs of these chromosomes were characterized by subterminal singles of 5S rDNA (red); the chromosomes were identified by Said et al. (2018) as 1P and 5P, respectively. **c**) The 45S rDNA (green) was detected on the translocated *A. cristatum* chromosome arm (arrows) in the background of wheat, while 5S rDNA (red) was absent. **d**) GISH using DNA from *A. cristatum* (green) distinguished the translocated arm (arrows). The chromosomes were counterstained with DAPI (blue). Scale bar is 10 µm.

Online Resource 2 (File name: ESM_2) containing Tables:
Supplementary Table S1 Frequency (%) of plants with various chromosome composition in progenies of wheat CS-*A. cristatum* lines (based on summarized data from chromosome counting and FISH).

Supplementary Table S2 Mean values for spike agronomic traits comparing Chinese Spring (CS) and wheat-*A. cristatum* addition and translocation lines

Online Resource 3 (File name: ESM_3) containing Supplementary Data:
Supplementary Data S1 COS markers used in the present study at the University of Córdoba and in the Hungarian Academy of Sciences together with their primer sequences and annealing temperatures. Detailed information for the PCR conditions has been given in the 'Materials and Methods'

Online Resource 4 (File name: ESM_4) containing Supplementary Data:
Supplementary Data S2 Source ESTs of the COS markers assigned to the P-genome chromosomes of tetraploid *A. cristatum*.

Online Resource 5 (File name: ESM_5) containing Supplementary Data:
Supplementary Data S3 Results of BLASTn search for COS markers assigned to tetraploid *A. cristatum* chromosomes in the reference sequences of hexaploid wheat chromosomes (www.wheatgenome.org/) and the start positions (bp) of the marker-specific ESTs. The EST source sequences were used as queries in BLASTn searches against the reference pseudomolecules of each wheat chromosome.

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Fig. 1 GISH on mitotic metaphase plates of wheat-*A. cristatum* lines using genomic DNA from tetraploid *A. cristatum* (green). **a**) Chromosome 4P disomic addition- **b**) chromosome arm 4PS ditelososomic addition- and **c**) homozygous translocation- 1PS·1BL lines. Chromosomes were counterstained by DAPI (blue). Bars = 10 µm

Fig. 2 The breeding procedure used in this study to obtain the ditelosomic addition chromosome short arm 3PS from tetraploid *A. cristatum* in the genetic background of wheat CS

Fig. 3 Spike morphology of wheat-*A. cristatum* chromosome (1P - 6P) disomic addition lines (a), ditelosomic addition lines (b) and translocation 1PS·1BL (c) in CS. Bars = 5 cm

Fig. 4 PCR amplification profiles used for the location of COS molecular markers on chromosomes 1P, 4P and 5P. **a-b)** COS617 and COS632 mapped on the short and long arms of chromosome 1P, respectively; **c-d)** COS087 and COS021 mapped on the short and long arms of chromosome 4P, respectively; **e-f)** COS108 and COS150 mapped on the short and long arms of chromosome 5P, respectively

Comentado [Dori9]: Change the code of these markers

Fig. 5 Visualization of wheat-*A. cristatum* orthologous regions from the perspective of wheat homoeologous chromosome groups I - VI (Group VII was omitted from this study due to the unavailability of a wheat-*A. cristatum* addition line for chromosome 7P). Physical map of the source ESTs of the COS-markers (right), the genomic positions on wheat pseudomolecules (kb) are on the left. Arrows indicate the centromere. S and L refer to the short and long arm, respectively (please refer to the online version for higher resolution)