Szent István University

Genome analysis of wheatgrass species using molecular cytogenetic tools and molecular markers

Doctoral (PhD) thesis

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1. BACKGROUND AND AIMS

Wheat (*Triticum aestivum* L., 2n=6x=42; BBAADD) is one of the most important cereal crop in the world, it is our second most important food crop after rice. A predicted world population will be over 9 billion by 2050 (FAO, 2009). Therefore global annual yield increases must rise from the current 1% to 1.6% (Giraldo et al., 2019). Maintaining this yield increase can be difficult because of the shrinking growing areas and the various abiotic and biotic stresses which could reduce the yield. Beside the application of different agricultural techniques, breeding new cultivars with high biotic and abiotic stress resistance, and/or high yield productivity can increase the crop yield. Wild relatives of wheat can be used for improving the stress resistance of wheat. Wild gene variants can be transferred by interspecific or intergeneric hybridization in order to increase the allelic diversity of wheat. (Hoffmann, 2011).

The wheatgrass species (the genus *Thinopyrum, Agropyron,* and *Pseudoroegneria*) are highly tolerant against abiotic stress, such as low temperature, drought and salinity, and resistant to several diseases, such as leaf and stem rusts, and powdery mildew (Wang, 2011). The efficiency of the gene transfer depends on the genetic relationship of the species, and the homeology between the wheat and alien chromosomes. Many resistance genes were identified in wheatgrass species and transferred into wheat genome by interspecific hybridization.

The final goal of interspecific or intergeneric hybridization is to produce stable wheat introgression lines carrying only the useful target genes from the wild species without any negative effect. For the successful gene transfer it is important to understand the genome structure of wheatgrasses and we need to identify their chromosomes using detailed FISH based karyotypes or chromosome specific molecular markers. *In situ* hybridization using labelled total genomic DNA as probes (genomic *in situ* hybridization, GISH) allows the visualization of alien chromosomes and chromosome segments. The individual chromosomes of a species can be identified by the hybridization pattern of repetitive DNA probes after fluorescence *in situ* hybridization (FISH). The application of molecular markers is a suitable tool to facilitate the wheat breeding programs through the high throughput selection of desirable lines carrying a small target gene.

The aims of our work was as follows:

- Improvement and optimization of molecular cytogenetic methods (GISH, FISH) to identify chromosomes and chromosome segments of wheatgrasses in wheat background, and the characterization of the alien chromosomes.

- Development of a detailed FISH based karyotype of three diploid wheatgrass species (*A. cristatum, Th. bessarabicum, Ps. spicata,*), and analysing the karyotypic polymorphisms between their accessions of different geographical origin.
- Exploring the genome relationship between *A. cristatum, Th. bessarabicum, Th. elongatum, Ps. spicata* and the wheat using molecular markers, and identifying genome specific molecular markers.
- Identifying chromosome specific molecular markers to the E-genome chromosomes of *Th. elongatum*, using PCR with DNA from a wheat-*Thinopyrum elongatum* amphiploid, a complete set of wheat-*Th. elongatum* disomic addition lines, and 12 ditelosomic addition lines.
- Studying the orthologous relationships between the chromosomes of *Th. elongatum* and bread wheat.
- Production of new wheat pre-breeding materials containing agronomical useful traits from *A. cristatum* or *Th. elongatum*.

2. MATERIALS AND METHODS

2.1. Plant material

The following diploid wheatgrass species were used for molecular cytogenetic and marker analysis: *Agropyron cristatum* (MvGB 1521, MvGB 1509), *Thinopyrum bessarabicum* (MvGB 1705, MvGB 1703, MvGB 1706), *Pseudoroegneria spicata* (MvGB 1607, MvGB 1617, MvGB 1615), *Thinopyrum elongatum* (MvGB 1708).

A wheat (Chinese Spring, CS)-*Thinopyrum elongatum* amphiploid, a complete set of wheat-*Th. elongatum* disomic addition lines, and 12 ditelosomic addition lines were used to identify chromosome specific molecular markers to the E-genome chromosomes of *Th. elongatum* using PCR.

In the crossing programs were carried out on the following lines: the CS- *A. cristatum* disomic addition lines, the previously mentioned CS- *Th. elongatum* addition lines, the CS- *A. cristatum* 5P disomic and 2C monosomic, and the CS- *A. cristatum* 6P disomic and 2C monosomic addition lines.

2.2. Molecular cytogenetic analysis

2.2.1. Chromosome preparation

The grains were germinated on wet filter paper, then the root tips 1-1,5cm long were collected, and incubated in ice water for at least 24 hours. To fixing the cell divison the cold-treated roots were transferred to 3:1 v/v mixture of absolute ethanol and glacial acetic acid, and incubated for 5 days at 37°C, followed by staining in acetocarmine for 2 hours at room temperature (RT). The grains after the collection of the root tips were planted in individual planting pots with tracking number. The root tips were stored at -20°C until using. Chromosome preparations were obtained using the squash method in 45v/v% acetic acid. The slides were investigated by light microscope, the coverslips were removed in liquid N_2 from the suitable slides, and dehydrated in a 70%, 90% and 100% ethanol series, dried for 1 day at RT, then stored at -20°C for weeks.

2.2.2. Fluorescence in situ hybridisation (FISH)

In order to identify the wheat and wheatgrasses chromosomes the following repetitive sequences were used: pTa71, pSc119.1, Afa-family, HT100.3, and pAs1, and some microsatellite probes: $(GAA)_n$, $(CAC)_n$, $(AGG)_n$, $(ACT)_n$, $(ACG)_n$, $(AAC)_n$, $(CAG)_n$. The pTa71 (Gerlach and Bedbrook, 1979) 9.05 kb long 18S fragment was amplified from rice, according to Chang et al. (2010). The pSc119.1 probe is a 120 bp long rye subtelomeric heterochromatic sequence (Bedbrook et al., 1980), was amplified from rye genomic DNA according to Contento et al. (2005). The 1 kb long pAs1 repetitive sequence was isolated from *Ae. tauschii* genomic DNA (Rayburn és Gill, 1986), digesting it with restriction endonuclease resulting the 260 bp long Afa-family sequence according to Nagaki et al. (1995). The probe HT100.3 was amplified from *Arabidopsis thaliana* DNA specific to telomeric region (Juchimiuk-Kwasniewska et al., 2011). The microsatellite probes $(GAA)_n$, $(CAC)_n$, $(AGG)_n$, $(ACT)_n$, $(ACG)_n$, $(AAC)_n$, $(CAG)_n$ were isolated from wheat genomic DNA according to Molnár et al. (2011). The probes Afa-family, pSc119.2, pTa71, pAs1, HT100.3 and the microsatellite probes were labelled by PCR with biotin-16-dUTP, and digoxigenin-11-dUTP. In the case of pSc119.2, pTa71, HT100.3, and the microsatellite probes the Biotin-Nick Translation Mix (Roche) was used, while the Afa-family, pAs1, pTa71 and the microsatellite probes the Dig-Nick Translation Mix (Roche) was used according to the manufacturer. The pre-hybridisation washes and the detection of the hybridisation signal were performed according to Molnár et al. (2011).

2.2.3. Genomic in situ hybridisation (GISH)

For GISH total genomic DNA was extracted from the following species: *A. cristatum, Th. elongatum, Th. bessarabicum,* and *Ps. spicata*. The total genomic DNA was labelled indirectly with biotin-14-dCTP by random priming (InVitrogen, BioPrime Random Labeling Kit) according to manufacturer. The pre-hybridisation washes and the detection of the hybridisation signal were performed according to Molnár et al. (2011).

2.3. COS marker analysis

For analysing the genomic relationship between *A. cristatum, Th. bessarabicum, Th. elongatum, Ps. spicata* and the wheat, and identifying chromosome specific molecular markers to the E-genome chromosomes of *Th. elongatum* 114 COS markers were used. The markers potentially covering the wheat homoeologous groups I-VII were chosen from publicly available COS marker collections (Wheat Genetic Improvement Network (WGIN) (http://www.wgin.org.uk/resources/Markers/TAmarkers.php), and Tools and Resources (TR) collections (http://www.modelcrop.org/cos_markers) (Howard et al., 2011; Quraishi et al., 2011). The PCR amplicons were separated with a Fragment Analyzer™ Automated CE System equipped with a 96-Capillary Array Cartridge (Advanced Analytical Technologies, Ames, USA) and the results were analysed and visualized with PROSize v2.0 software.

2.4. Sequence analysis

Analysing the chromosomal synteny between bread wheat and diploid *Th. elongatum* the source EST sequences of the COS markers were aligned with the Chinese Spring reference sequence v1.0 using BLASTn algorithm (https://urgi.versailles.inra.fr/blast/). Positions of the first basepair (bp) of the best hit in the wheat pseudomolecule was used as physical positions of each COS marker. The lengths (in bp) of the wheat pseudomolecules and the start genomic positions of the ESTs were converted to pixels and physical maps of the COS markers were designed using custom-made software (http://geneticmap.herokuapp.com/). To visualize the wheat-*Th. elongatum* syntenic relationships, the markers on the wheat physical map were colour-coded to show the chromosomal locations in *Th. elongatum*.

2.5. Artificial leaf rust inoculation

Leaf rust inoculation was carried out in greenhouse on the CS-*A. cristatum,* and on the CS-*Th. elongatum* disomic and ditelosomic addition lines. The inoculation was on the 8th day after

the sowing with a suspension of uredospores. The severity of disease symptoms were evaluated on the 10th day after the inoculation using a scale according to Stakman et al. (1962).

3. RESULTS

3.1. Karyotypic analyis

FISH karyotypes of two accessions of *Agropyron cristatum* (MvGB 1509, MvGB 1521), three accessions of *Thinopyrum bessarabicum* (MvGB 1703, MvGB 1705, MvGB 1706), and two accessions of *Pseudoroegneria spicata* (MvGB 1615, MvGB 1607) with different geographical origins were generated and the FISH polymorphism were analysed using the pSc119.2, the Afa-family and the pTa71 repetitive DNA probes. All the chromosomes could be distinguished according to their FISH signals. All the microsatellites probes failed to show any signals on the wheatgrasses genomes, unlike in the case of other *Triticeae* species (bread wheat, *Aegilops, T. monococcum, Secale*). The lack of these trinucleotide sequences is not unusual for the wheatgrass species. The chromosomes of *Th. elongatum* didn't showed any hybridisation signals using microsatellite probes (Linc et al., 2012), and in the case of *Th. ponticum* only the chromosome 7 showed GAA signal in the centromeric region (Sepsi et al., 2008). The chromosomes of *Ps. spicata* showed the most complex hybridisation pattern, while the chromosomes of the *A. cristatum* showed the simplest pattern, but it was suitable for the identification. There were no detectable FISH polymorphism between the three accessions of *Th. bessarabicum* with different geographical origin, while the chromosomes of *A. cristatum* and *Ps. spicata* showed some major polymorphism.

3.2. COS marker analysis

3.2.1. Phylogenetic relationships between wheatgrass species and wheat based on COS markers

The hexaploid bread wheat (GK Öthalom), the *Th. elongatum*, the *Th. bessarabicum*, two accessions of *A. cristatum*, and three accesions of *Ps. spicata* genomes were analysed using COS markers. Out of the 112 loci (82 markers) detected in the E-genome of diploid *Th. elongatum* 53 (43 markers) showed significant length polymorphism (>5bp) relative to the wheat genotype GK Öthalom. In the case of *Th. bessarabicum*, where 109 loci (82 markers) were located on the J-genome, 52 loci (44 markers) were polymorphic. In *Ps. spicata* genotypes MvGB1607, MvGB1615 and MvGB1617, 118, 135, and 113 loci, respectively, were assigned to the St genome, of which 57 markers (76 loci), 62 markers (87 loci) and 56 markers (71 loci) showed significant size polymorphism. Finally, 57 markers (73 loci), and 62 markers (82 loci)

were polymorphic among the 89 located on *A. cristatum* genotypes MvGB1521 and MvGB1509 (P-genome), respectively.

The dendrogram generated from the analysis of similarity separated most of the populations and all the species, which split into three groups. The first group included the *Th. elongatum*, the *Th. bessarabicum* and *T. aestivum*. Both genotypes of the P-genome species *A. cristatum* fell in the second group, while the three genotypes of the St-genome species *P. spicata* formed a third, distantly separated clade.

3.2.2. Identifying chromosome specific markers to the *Thinopyrum elongatum* (EE)

A complete set of wheat-*Th. elongatum* disomic addition lines, and 12 ditelosomic addition lines were used to identify chromosome specific molecular markers to the E-genome chromosomes of *Th. elongatum.* Out of the selected 114 COS markers 108 (94.7%) showed PCR products in at least one of the genotypes. Out of the 108 markers 50 showed size polymorphism (≥4bp) between the amphiploid line and the parental wheat genotype CS. Thirtyone (62%) of these 50 markers could be assigned to a single E-genome chromosome on the basis of chromosome addition lines, nine markers specific for the w3 chromosomes could be mapped to the amphiploid but not to any ofthese chromosomes, suggesting these markers are located on the missing 3E chromosome. In total 40 chromosome specific marker could be assigned: 1E:4; 2E:3; 3E:9; 4E:8; 5E:5; 6E:5; 7E:6.

3.2.3. Sequence analysis

In order to compare the structure of the E genome of *Th. elongatum* with the A, B and D genomes of bread wheat, the source EST sequences of the 50 COS markers producing polymorphic amplicons on the E-genome chromosomes were aligned to the IWGSC v1.0 reference sequences of the wheat chromosomes. To do this, a BLASTn sequence similarity search was made for source EST sequences against the IWGSC wheat pseudomolecules. Based on the start positions of the aligned sequences of the best hits (E-value < $2.8e^{-08}$, Identity > 82% and sequence length > 100bp), a physical map was constructed for all the chromosomes of wheat homoeologous groups 1–7. Most of the markers were located on the same homoeologous group chromosomes in *Thinopyrum* as in wheat, four markers were located on non-homoeologous chromosomes in *Thinopyrum* relative to wheat. The chromosomal location ofCOS markers revealed a large-scale chromosome rearrangement and several intragenomic duplications in the *Thinopyrum elongatum* chromosomes. Six markers specific for the group 2 chromosomes ofwheat detected a large-scale chromosome rearrangement involved in 2EL and 6ES. The TR636 marker was located in both 6ES and 6EL suggesting an intrachromosomal

rearrangements. Some chromosomal rearrangements were identified: a 4ES/5EL, and a 5EL/7ES duplications. The location ofthe COS markers on the chromosome arms ofThinopyrum and wheat allowed wheat-*Thinopyrum* macrosyntenic relationships to be visualized at the chromosomal arm level. The marker content of the short and long arms of the w1 and w2 chromosomes were mapped on the homoeologous chromosome arms of the E genome. The same was observed for the short arm of group 6 chromosomes of wheat and, to a lesser extent, for the group 7 chromosomes.

3.3. Artificial leaf rust inoculation

The leaf rust resistance of CS, CS-*A. cristatum,* and CS-*Th. elongatum* disomic (DA) and ditelosomic (DtA) addition lines were evaluated in greenhouse by artificial leaf rust infection at seedling stage. All of the CS-*Th. elongatum* lines were susceptible (Stakman-scale: 4) to leaf rust. In the case of CS-*A. cristatum* addition lines we could identify some resistant line. The DA2PL was very resistant (;), the DtA2PS was susceptible (4), while the DA2P was found to be moderately susceptible (3). The DtA1PS could be both resistant (1), or moderately resistant (2), but DA1P was found to be moderately resistant (2), DtA1PL was not evaluated, because we did not have it. The DtA6PS was moderately susceptible (3), the DtA6PL was susceptible (4), the DA6P showed moderately resistance (2). All of the other lines (DA: 3P, 5P; DtA: 4PS, 5PL) were susceptible (4). The DtA3PS germinated poorly, and the plants were weak and died before inoculation, the DA4P plants died soon after the inoculation.

3.4. Production of new prebreeding materials

The first crossing program was carried out between the CS- *A. cristatum* the CS- *Th. elongatum* disomic and monosomic addition lines. The inheritance of chromosomes 1E and 1P was stable, they were found in every progeny lines. The inheritance of chromosome 3E was higher than chromosome 3P, which usually eliminated or broken. The chromosome 2P was very instable so we could not make crossing with them. In total we could detect one line containing translocation.

In the second crossing program we analysed the F_2BC_1 generation from the cross CS-*A. cristatum* disomic addition lines and CS-*Ae. cylindrica* 2C disomic addition lines with gametocidal effect. In these generations could be expected some chromosomal rearrangements caused by the gametocidal genes, and we selfpollinated these generations in order to produce further rearrangements. We could detect in total two lines containing 6P and six lines containing

5P translocation. Seeds were developed in all of the translocation lines, which can be used to stabilize the translocations in disomic form, and recover the wheat background by multiple backcrossing.

3.5. New scientific results

1. The karyotypes of two accessions of *A. cristatum* (MvGB1509, MvGB1521), three accessions of *Th. bessarabicum* (MvGB1705, MvGB1703, MvGB1706) and two accessions of *Ps. spicata* (MvGB1615, MvGB1607) were generated using repetitive DNA sequences: Afa-family, pSc119.2 and pTa71. All the chromosomes of the diploid species could be distinguished with them. These karyotypes can be used to identify the chromosomes of *Th. bessarabicum* and traced in the prebreeding programs.

2. We could confirm the difference between *Th. elongatum* (E) and *Th. bessarabicum* (J) according to the generated karyotype and the result of COS marker analysis.

3. Analysing the genomes of *Th. elongatum, A. cristatum, Ps. spicata* and *Th. elongatum* using COS markers showed that the *Th. elongatum* and *Th. bessarabicum* are more closely related to hexaploid wheat than *A. cristatum*, while *Ps. spicata* is the most distantly related genome. These results are consistent with the outcome of our crossing programs. We found that the inheritance of the chromosomes of *Th. elongatum* is higher than the chromosomes of *A. cristatum*.

4. We identified some chromosome specific COS markers to the E genome using CS-*Th. elongatum* disomic and ditelosomic addition lines. With these markers we analysed the syntenic relationship of wheat and *Thinopyrum* chromosomes. The markers could be useful to identify wheat-*Thinopyrum* introgression lines in the future.

5. Leaf rust resistance was detected on the chromosome 2P of *Agropyron cristatum* by artificial infection of the CS-*A. cristatum* addition lines.

6. Two translocation lines containing 6P and six translocation lines containing 5P chromosomes were identified by crossing CS- *A. cristatum* and CS-*Ae. cylindrica* 2C disomic addition lines. All of the lines developed seeds which can be useful as prebreeding material.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Identification of wheatgrass chromosomes by molecular cytogenetic methods

Detailed FISH based karyotypes of genomes *Th. bessarabicum, A. cristatum, Ps. spicata* were created using repetitive DNA probes Afa-family, pSc119.2, and pTa71. Based on these karyotypes all the chromosomes could be identified, and some polymorphism were detected between accessions from different geographical origin. Using these karyotypes will be possible to identify and select new pre-breeding materials. Increasing the number of diagnostic bands is necessary in order to identify smaller rearrangements, identifying more repetitive DNA probes, or designing single-gene FISH probes should be advisable.

4.2. COS marker analysis

The COS marker analysis had two part. First we analysed four wheatgrass species (*A. cristatum, Ps. spicata, Th. elongatum, Th. bessarabicum*) using more than 100 COS markers. The phylogenetic relationship between these species and bread wheat was analysed. Beside we identified specific markers for all seven E genome chromosome which markers can be useful for marker assisted selection (MAS) in wheat pre-breeding programs, selecting addition and translocation lines. We are planning to identify and design more markers specific to the E genome and to other wheatgrass genomes.

4.3. Artificial leaf rust inoculation

Artificially inoculating seedlings of CS-*A. cristatum* addition lines resulted in the high level of resistance of the long arm of the 2P chromosome. Leaf rust and powdery mildew resistance genes were already mapped on this chromosome. Based on these results it would be beneficial to crossing this line with *ph1b* mutant wheat line in order to produce recombinants which contains this resistance gene. If this crossing would be successful the resistance gene could be identified by MutChromSeq method.

4.4. Production of new prebreeding materials

Our crossings were aimed to develop translocation lines which contains useful genes from both E and J genomes. We crossed wheat-*Th. elongatum* and wheat-*A. cristatum* disomic addition lines and analysed the F_1 generation. We were able to identify one plant containing

translocation. We also completed a crossing program using gametocidal system, and analysed the progenies from the crossings of wheat-*Ae. cylindrica* 2C and wheat-*A. cristatum* disomic addition lines. We identified 8 translocation lines containing 5P and 6P chromosomes using molecular cytogenetic methods (FISH, GISH). The identified translocation lines are considered as promising pre-breeding materials which need further detailed analysis.

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