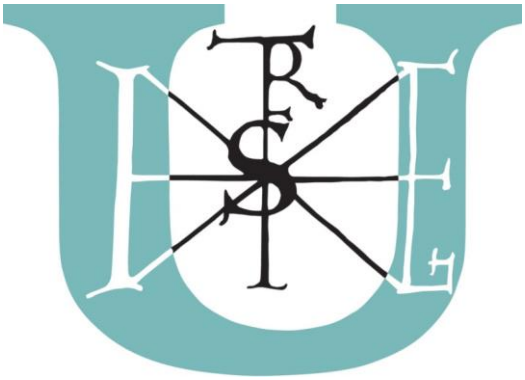

Szent István University

Development, phenotypic and genotypic characterization of wheat–*Aegilops* introgression lines

András Farkas

Gödöllő

2018

Doctoral School: Doctoral School of Plant Science

Branch of Science: Agricultural and Horticultural Sciences

Head of School: Prof. Lajos Helyes, D.Sc.
Head of Department of Horticulture and Technology
Szent István University

Supervisor: István Molnár, Ph.D.
Senior Research Associate
Centre for Agricultural Research, Hungarian Academy of Sciences, Agricultural institute

…… ……

Lajos Helyes, D.Sc. István Molnár, Ph.D.

Head of Doctoral School Supervisor

1. BACKGROUND AND AIMS

Wheat (*Triticum aestivum* L.) is one of the most important food crop in the world and contributes about 20% of the total dietary calories consumed by people worldwide. (Braun et al. 2010). Domestication and plant breeding presumably narrowed the genetic diversity of bread wheat, which could limit the future crop improvement against various pathogens and unfavourable trend of the climatic change.

One of the possible method for improving the stress resistance is the transfer of useful agronomic traits from wild relatives into wheat by interspecific or intergeneric hybridisation. These wild species haven't been breeded so they represent a rich source of genes and gene complexes that can be utilized in wheat improvement via chromosome-mediated gene transfer.

Goatgrasses (*Aegilops*) are the closest relatives of *Triticum*. The wide distribution area indicates the good adaptation ability of the genus. *Aegilops* species have considerable genetic variability, therefore they are excelent sources for stress resistance and biofortification of wheat. Beside the fact that several useful traits have been transfered from *Aegilops* species to wheat, their genetic diversity is largely underutilised. In order to promote the efficiency of introgression breeding, it is important to improve our knowledge from their genome structure, to develop new genome specific molecular tools and to identify genes responsible for useful agronomical traits.

During the chromosome mediated gene transfer from a wild species to wheat we produce F_1 hybrids, amphiploids, than additions, substitutions and finally translocation lines.

In the last decade some wheat–*Aegilops* addition lines (1U, 3U, 2M, 3M, 7M) were produced using the *Ae. biuncialis* accession MvGB642 in

Martonvásár, but this set is still incomplete. In order to produce complete set of addition lines and to transfer of additional traits, two other *Ae. biuncialis* accessions (MvGB382, MvGB1112) have been involved in the crossing programmes. Tan et al. (2009) produced a wheat–*Ae. biuncilais* partial amphiploid, later Zhou et al. (2014) selected an $1U^b$ addition line. In the Department of Genetic Resources Molnár (2008) developed a $3M^b$ substitution and a $3M^b$ centric fusion with the *Ae. biuncialis* MvGB642 genotype. One of our aims was a detailed molecular cytogenetic characterization (FISH), investigation of agronomical traits and the micronutrient content of these lines.

Thanks to their fertility, the synthetic amphyploids are considered as valuable prebreeding material. With their help, several pest resistance genes have been transfered from the wild relatives into the bread and durum wheat.

We resolve to made synthetic amphiploids with the cross of durum wheat with *Ae. umbellulata* and *Ae. uniaristata.*

In the course of prebreeding it is important to detect and identify the alien chromosomes in the wheat background. Fluorescence *in situ* hybridization (FISH) with repetitive DNA probes is a powerful method to produce specific hybridization patterns allowing the identification of the chromosomes. In the tribe of the *Triticeae* the most commonly used probes are the Afa family, pSc119.2 and pTa71. In the case of some *Aegilops* chromosomes these probes did not give frequent bands in the interstitial region. Simple sequence repeats (SSRs) or microsatellites are a widespread and highly abundant class of tandemly repeated DNA sequences within the *Triticeae* and *Aegilops* genomes. Some of them have been used in the molecular cytogenetic studies of wheat and barley. The description of the karyotypes with different SSR probes on the *Aegilops*

chromosomes could be used for the identification of the chromosomes, chromosome segments and chromosome rearrangements in wheat–*Aegilops* introgression lines.

The aims of our work was as follows:

- Analysis of the chromosomal distribution of $(GAA)_n$, $(ACG)_n$, $(CAG)_n$, $(AAC)_n$, $(CAC)_n$ and $(ACT)_n$ SSR clusters on *Ae. umbellulata*, *Ae. comosa*, *Ae. uniaristata*, *Ae. tauschii*, *Ae. speltoides* and *Ae. markgrafii* chromosomes using fluorescent *in situ* hybridization
- Selection of new wheat–*Ae. biuncialis* prebreeding material
- Selection of wheat–*Ae. biuncialis* translocation lines
- The molecular cytogenetic identification of the wheat–*Ae. biuncialis* MvGB642 $3M^b$ centric fusion and investigation of its agronomical traits and micronutrient content
- Production of durum wheat (*Triticum turgidum* subsp. *durum*)–*Ae.umbellulata* and *Ae. uniaristata* synthetic amphiploids.

2. MATERIALS AND METHODS

2.1. Plant materials

The following *Aegilops* species were used for the FISH karyotyping with SSR probes: *Ae. comosa* (TA2760), *Ae. markgrafii* (MvGB428), *Ae. speltoides* (MvGB905), *Ae. tauschii* (MvGB605), *Ae. umbellulata* (AE740/03) and *Ae. uniaristata* (JIC2120001).

‘Mv9kr1’, *Ae. biuncialis* MvGB642, *Ae. biuncialis* MvGB382 and *Ae. biuncialis* MvGB1112 genotypes were used for the development of wheat–*Ae. biuncialis* introgression lines.

Grain micronutrient analysis was carried out on the wheat–*Ae. biuncialis* $3M^b$ addition line (Schneider et al. 2005), wheat–*Ae. biuncialis* $3M^b$(4B) substitution line, wheat–*Ae. biuncialis* $3M^b$.4BS centric fusion (Molnár 2008), *Ae. biuncialis* MvGB642, *Ae. biuncialis* MvGB382 and ’Mv9kr1’.

T. turgidum subsp. *durum*’GK Novodur’, *Ae. umbellulata* AE740/03 and *Ae. uniaristata* JIC2120001 was used in the development of synthetic amphiploids.

2.2. Plant growing and crosses

The plants were grown and the crosses were made in the field nursery (Martonvásár, Tükrös), in phytotron growth chambers and in the glasshouse. Spikes of the female parents were emasculated and pollinated 2-4 days later using spikes cut from male parents.

The *T. turgidum* subsp. *durum* ’GK Novodur’ × *Ae. umbellulata* AE740/03 and the *T. turgidum* subsp. *durum* ’GK Novodur’ × *Ae. uniaristata* JIC2120001 F_1 plants were threated with colchicine.

2.3. Molecular cytogenetic analysis

2.3.1. Genomic *in situ* hybridisation (GISH)

In order to visualise the *Aegilops* genomes in the wheat background, DNA was isolated from *Ae. comosa, Ae. tauschii, Ae. umbellulata, Ae. uniaristata.* Total genomic DNA was labelled indirectly with digoxigenin-11-dUTP and biotin-14-dCTP by random priming. The

prehybridization washes and the detection of the hybridization signals were performed according to Molnár et al. (2011).

2.3.2. Fluorescent *in situ* hybridisation (FISH)

Afa family, pSc119.2 and pTa71 probes were hybridized to mitotic metaphase chromosome preparation in order to identify the wheat and *Aegilops* chromosomes. The pSc119.2 and Afa-family sequences were amplified and labelled with biotin-14-dATP and digoxigenin-11-dUTP, respectively, using PCR (Nagaki et al. 1995; Contento et al. 2005). The pTa71 (Gerlach és Bedbrook 1979) 18S fragment was amplified from rice, according to Chang et al. (2010). It was labelled with 50% biotin-14-dATP and 50% digoxigenin-11-dUTP. The $(GAA)_n$, $(ACG)_n$, $(CAG)_n$, $(AAC)_n$, $(CAC)_n$ and $(ACT)_n$ microsatellite probes were amplified with PCR and labelled with digoxigenin-11-dUTP and biotin-14-dATP using nick translation. The prehybridizition washes and the detection of the hybridization signals were performed according to Molnár et al. (2011).

2.3.3. SSR marker analysis

The microsatellite (SSR) markers specific for the 4BS (*Xbarc1045*, *Xgwm113*, and *Xgwm368*) and 4BL (*Xgwm149*, *Xgwm251*, and *Xgwm165*) chromosome arms (Röder és mtsai. 1998, Somers és mtsai. 2004) were used to characterize the $3M^b$ addition, $3M^b$ substitution, $3M^b$ centric fusion, 'Chinese Spring'*ph1b* mutant and the *Ae. biuncialis* MvGB642 plants.

2.4. Artificial leaf rust inoculation

Leaf rust inoculation carried out in greenhouse on the *T. turgidum* subsp. *durum* 'GK Novodur' × *Ae. uniaristata* JIC2120001 and *T. turgidum* subsp. *durum* 'GK Novodur' × *Ae. umbellulata* AE740/03 amphiploids.

Severity of disease symptoms were evaluated using a scale (0, ;, 1-4) published by Stakman et al. (1962).

2.5. Micronutrient analysis

The micronutrient content in the grains of the $3M^b$ addition, $3M^b$ substitution, $3M^b$ centric fusion, parental lines ('Mv9kr1', *Ae. biuncialis* MvGB642) and the *Ae. biuncialis* MVGB382 were measured with atomic absorption spectrophotometer (Farkas et al. 2014).

2.6. Statistical analysis

The statistical analyses were carried out using the Excel for Windows program. The results are the means ± standard deviation of three measurements per genotype for micronutrient analysis and 10 measurements per genotype for morphological parameters. Differences between the genotypes and the wheat parental line Mv9kr1 were determined by means of Student's t test for paired data at the $P = 0.05$ and $P = 0.01$ significance levels.

3. RESULTS

3.1. Application of simple sequence repeats (SSR) as FISH probes

We described the karyotypes of *Ae. umbellulata* AE740/03, *Ae. comosa* TA2760, *Ae. uniaristata* JIC2120001, *Ae. tauschii* MvGB605, *Ae. speltoides* MvGB905 and *Ae. markgrafii* MvGB428 accessions with the standard repetive probes pSc119.2, Afa family and pTa71. These karyotypes served as reference for the identification of all the chromosomes of *Aegilops* species. After the hybridization with the standard FISH probes the signals were wash down and rehybridized with $(GAA)_n$, $(ACG)_n$, $(CAG)_n$, $(AAC)_n$, $(ACT)_n$, and $(CAC)_n$ SSR probes. The

$(GAA)_n$ and $(ACG)_n$ probes produced the most bands, the exeption was the *Ae. tauschii* were these probes gave only a few signals. U, M, S and C genomes had the most complex hybridization pattern with the $(GAA)_n$ probe. Diagnostic bands were detected on the 3M, 4M, 1D, 2D, 2S, 2C, 3C, 6C and 7C chromosomes. The $(ACG)_n$ motif gave mainly pericentromeric bands on the U, M, N, S and C genomes. Diagnostic $(ACG)_n$ bands were detected on the a 4D, 4C, 7C, 4U, 3M, 4M, 7M, 2N and 7N chromosomes. Diagnostic $(CAG)_n$ signals were also detected on the 1N and 7N *Ae. uniaristata* chromosomes.

3.2. Production of wheat–*Aegilops biuncialis* introgression lines

We produced BC_2 and BC_3 generations backcrossing the wheat ('Mv9kr1') × *Ae. biuncialis* (MvGB642, MvGB382, MvGB1112) amphiploids. The progenies were analysed with molecular cytogenetic methods (FISH, GISH). We identified *Aegilops* chromosomes in the progenies from which addition line doesn't exist. We detected the 2U, 4U, 5U, 6U, 6M chromosomes and 1DL.1DS-U, 5DS.5DL-M, 4DS.4DL-M, 3DS.3DL-M, ML.MS-wheat translocations in the 'Mv9kr1' × *Ae. biuncialis* MvGB642 BC_3 progenies; 5U, 7U, 1M–7M chromosomes and the 3DS.3DL-U translocation line in the MvGB1112 BC_2 progenies and 1U, 2U, 4U, 5U, 6U, 6M and 7M chromosomes among the MvGB382 BC_2 plants.

3.3. Molecular cytogenetic characterization of the wheat–*Ae. biuncialis* MvGB642 $3M^b$ centric fusion and investigation of agronomical traits and micronutrient content

The karyotypic composition of the $3M^b$ substitution and $3M^b$ centric fusion was characterized by sequential FISH and GISH. The lines were classified as $3M^b$(4B) disomic substitution and 3Mb.4BS centric fusion.

SSR markers specific for the 4BS and 4BL chromosome arms were also used to confirm the cytological results. The *Ae. biuncialis* accessions (MvGB382 and MvGB642) showed significantly higher concentrations of K, Zn, Fe, and Mn than the parental wheat line 'Mv9kr1'. The grains of the $3M^b$.4BS centric fusion had higher Zn (+23.4%) and Mn content (+38.2%) than 'Mv9kr1'. The $3M^b$(4B) substitution line tended to have the poorest agronomical value, while the $3M^b$ addition was intermediate and the $3M^b$.4BS centric fusion was the best. This tendency was clearly manifested in the plant height, the length of the main spike, and the seeds per plant. In the case of the $3M^b$.4BS centric fusion, the tillering ability, the length of the main spike, and the spikelets per main spike were similar to those of the parental wheat genotype 'Mv9kr1'.

3.4. Production of durum wheat–*Aegilops* synthetic amphiploids

Durum wheat × *Ae. uniaristata* and durum wheat × *Ae. umbellulata* synthetic amphiploids were produced. The genome composition of amphiploid were characterized by GISH and FISH. It was proved that the 'GK Novodur' × *Ae. umbellulata* amphiploids were immune to artificial leaf rust inoculation in seedling stage.

3.5. New scientific results

1. The karyotypes of *Ae. umbellulata* AE740/03, *Ae. comosa* TA2760, *Ae. uniaristata* JIC2120001, *Ae. tauschii* MvGB605, *Ae. speltoides* MvGB905 and *Ae. markgrafii* MvGB428 accessions were described with the standard repetitive probes Afa family, pSc119.2 and pTa71. All the chromosomes of the diploid species could be discriminated with them.

2. The chromosomal localization of SSR probes $(GAA)_n$, $(ACG)_n$, $(CAG)_n$, $(AAC)_n$, $(ACT)_n$, and $(CAC)_n$ were described on *Ae. umbellulata* AE740/03, *Ae. comosa* TA2760, *Ae. uniaristata* JIC2120001, *Ae. tauschii* MvGB605, *Ae. speltoides* MvGB905 and *Ae. markgrafii* MvGB428. The $(GAA)_n$ and $(ACG)_n$ probes produced the most bands, they can use potentially for the detection of *Ae. comosa*, *Ae. tauschii*, *Ae. markgrafii* and *Ae. uniaristata* chromosomes.

3. 'Mv9kr1' × *Ae. biuncialis* (MvGB642, MvGB382, MvGB1112) BC_2 and BC_3 plants were produced. Among the 'Mv9kr1' × *Ae. biuncialis* MvGB642 BC_3 progenies 2U, 4U, 5U, 6U, 6M chromosomes, and 1DL.1DS-U, 5DS.5DL-M, 4DS.4DL-M, 3DS.3DL-M, ML.MS-wheat translocation were detected, among the MvGB1112 BC_2 plants 5U, 7U, 1M–7M and 3DS.3DL-U translocation, in the MvGB382 BC_2 generation 1U, 2U, 4U, 5U, 6U, 6M and 7M chromosomes were identified.

4. The $3M^b$ substitution and centric fusion were classified as $3M^b$(4B) disomic substitution and 3Mb.4BS centric fusion with the help of *in situ* hybridization and SSR markers.

5. The grains of *Ae. biuncialis* accessions (MvGB382 and MvGB642) showed significantly higher concentrations of K, Zn, Fe, and Mn than the wheat line Mv9kr1.

6. The grains of the $3M^b$.4BS centric fusion had higher Zn and Mn content than the wheat parent 'Mv9kr1'.

7. *T. turgidum* subsp. *durum* 'GK Novodur' × *Ae. uniaristata* and *T. turgidum* subsp. durum 'GK Novodur' × *Ae. umbellulata* synthetic amphiploids were produced and the genome composition was characterized. The durum wheat × *Ae. umbellulata* amphiploids were immune to leaf rust inoculation in seedling stage.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Application of simple sequence repeats (SSR) as FISH probes

The SSRs as FISH probes increased the diagnostic bands on *Aegilops* chromosomes. In some cases we can not identified the *Aegilops* chromosome segments in the translocation lines orginated from the wheat × *Ae. biuncalis* crosses. For the detailed characterization of translocation lines the $(GAA)_n$ and $(ACG)_n$ probes can be used.

The chromosomes of *Aegilops* species which were involved in our study were previously isolated using flow cytometry exept the *Ae. uniaristata*. The $(GAA)_n$ and $(ACG)_n$ microsatellite repeat probes can be used to fluorescently label chromosomes in suspension prior to flow-cytometric analysis to facilitate sorting chromosomes of the *Ae. uniaristata*.

4.2. Production of wheat–*Aegilops biuncialis* introgression lines

In the last decade some wheat–*Aegilops* addition-, substitution- and translocation lines were produced but the genetic potential of the *Aegilops* species is underutilized. We developed BC_2 and BC_3 progenies from the 'Mv9kr1' × *Ae. biuncialis* (MvGB642, MvGB382, MvGB1112) amphiploids by backcross method. Many chromosomes which haven't been represented in the addition lines were identified. These genotypes are potential sources for the production of new wheat–*Ae. biuncialis*

addition lines. Selfing the monosomic translocation we can obtain them in a stable disomic form.

One of the relevant properties of the *Ae. biuncialis* MvG642 is the leaf rust resistance. The chromosomal localization of the resistance is still unknown, that's why we plan to check the resistance of the translocation lines and the newly developed progenies from the 'Mv9kr1' × *Ae. biuncialis* MvGB642 crossings.

Only a small number of cost-effective molecular markers specific for *Aegilops* chromosomes are available, which limits the high-throughput marker-assisted selection of introgression lines.

That's why we started to develop markers specific for the chromosomes of each of the seven homeologous groups in *Ae. biuncialis.* This marker design is based on a previous work, where all individual chromosomes of the diploid *Ae. umbellulata* were flow-sorted at high purity and they were sequenced (Illumina HiSeq2000). Wheat ESTs were selected than aligned to the *Aegilops* 1U, 2US, 2UL, 3U, 4U, 5U, 6U, 7U and 7UL chromosome sequences. Primers were designed to InDel regions. These markers specific for the U and M genome could promote the identification of wheat–*Ae. biuncialis* addition and translocation lines in the future.

4.3. The fluorescent *in situ* characterization of the wheat–*Ae. biuncialis* MvGB642 3M^{b} centric fusion and investigation of agronomical traits and micronutrient content

The genetic background of the 3M^{b}.4BS centric fusion have some disadvantageous traits because it has 'Chinese Spring' alleles too. In order to transfer the centric fusion to a modern wheat variety we started a

crossing program.'Mv Ménrót' was chosen as the recipient, which is an elit wheat cultivar, and has been cultivated since 2014.

Using the DArTseq method we genotyped a diverse *Ae. biuncialis* population and an *Ae. biuncialis* biparental mapping population. The mapping population will be used for the production of recombinant inbred lines which can be used for the QTL mapping of agronomic traits, including high micronutrient content. Genetic markers linked to the QTLs could be used for the targeted introgression desirable traits by marker assisted selection.

4.4. Production of durum wheat–*Aegilops* synthetic amphiploids

Thanks to their fertility, the synthetic amphyploids are considered as valuable prebreeding material. With their help, several pest resistance genes have been transfered from the wild relatives into the bread and durum wheat. We produced durum wheat x *Ae. uniaristata* and durum wheat x *Ae. umbellulata* amphiploids. The newly developed amphiploids can be considered as bridge materials for the chromosome mediated gene transfer into wheat. In order to transfer the usuful agronomical traits we crossed the amphiploids with the 'Chinese Spring' *ph1b* mutant to induce homeologous recombination beetween the wheat and *Aegilops* chromosomes.

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Cumulative impact factor: 28,547
Citations (independent/self): 85 (37/48)