



SZENT ISTVÁN UNIVERSITY

DEVELOPMENT OF NEW WHEAT/BARLEY AND  
WHEAT/*AGROPYRON* INTROGRESSSION LINES AND THEIR  
IDENTIFICATION USING FLUORESCENT *IN SITU* HYBRIDIZATION  
AND MOLECULAR MARKERS

*DOCTORAL (PHD) THESIS*

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## 1. BACKGROUND AND OBJECTIVES

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Among cereals, bread wheat (*Triticum aestivum* L.) is grown on the greatest land area in the world: the growing area extends over about 245-250 million hectares. According to predictions world population may grow to 9 billion by 2050; therefore, it would be crucial to increase the amount of crops by 2% annually to satisfy the population's food demand. This means an enormous challenge, both because of the negative effects of climate change and the shrinking growing areas. Besides the application of high quality agricultural techniques, increasing the crop amount and reducing yield losses after harvest could be achieved by breeding cultivars with high biotic stress resistance and higher productivity.

The genetic variability of bread wheat can be increased by the introgression of new genes and alleles from wild and cultivated species related to wheat, which provides rich gene pools for breeding. This kind of practice existed already at the end of the 19<sup>th</sup> century beginning with wheat/rye and wheat/wheatgrass crosses. However, a real breakthrough in the interspecific hybridization was brought by the development of colchicine treatment, which made it possible to produce fertile amphiploids by doubling the number of chromosomes. Later, the appearance of *in vitro* embryo rescuing techniques ensured the more widespread use of crosses between related species.

Besides corn and wheat, barley (*Hordeum vulgare* L.) is another important crop species, which is well adapted to abiotic stress conditions. Its cultivation requires the third greatest growing area among cereals. In our country barley has a multi-purpose utilisation: it serves as fodder crop and as basic material for beer and malt production, but it is also suitable for human consumption as pearl-barley. The wheat × barley crosses allow transferring different important

agronomic traits from barley into wheat like earliness, amino-acid and dietary fibre content, salt and drought tolerance and better tillering ability.

Numerous wild species of wheat carry agronomically valuable genes. The species of *Thinopyrum* (wheatgrass) genus are resistant to several biotic and abiotic stress factors. The natural populations of these species are successfully adapted to the most extreme climate conditions, thus their genetic variability was maintained. Several wheatgrass species have been already utilised for improving the biotic stress tolerance of bread wheat. Two of the most important gene pools of bread wheat from genus *Thinopyrum* are *Th. intermedium* and *Th. ponticum*, primarily for their resistance gene content (mainly against leaf rust, stem rust and powdery mildew) and abiotic stress tolerance (drought, alkaline soils) and secondly because their basic genomes (J and St) are closely related to the A and D genomes of wheat. Exploitation of the inherent potential of this species can be achieved by further crossings and selections.

Last but not least, the purpose of interspecific and intergeneric crosses is to develop stable inherited introgression lines, which, if it is possible, carry only useful genes from the wild relatives. For the identification of the introgressed alien chromosome segments, molecular cytogenetic methods and molecular markers are used. The fluorescent *in situ* hybridisation (FISH) using repetitive DNA probes allows the safe recognition of wheat and barley chromosomes based on their individual patterns, while identification of the chromosomes from *Thinopyrum* species needs the elaboration of new molecular cytogenetic techniques.

#### Aims:

- Selection of the 'Asakaze'/ 'Manas' wheat/barley ditelosomic addition lines, which, besides the whole wheat genome, carry only one chromosome arm pair from barley; molecular cytogenetic analysis of these lines; characterisation of

morphological and agronomic properties; investigation of salt stress tolerance of the lines;

- Improving the agronomic traits of the earlier developed 3HS.3BL translocation line by crosses with a modern Martonvásár wheat cultivar 'Mv Bodri', whose genetic material can be used later in pre-breeding programs;
- Introgression of resistance genes from *Agropyron gael* (hybrid of *Th. intermedium* and *Th. ponticum*) against the fungal diseases of bread wheat and development of wheat/*Agropyron glael* introgression lines.

## 2. Materials and Methods

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### 2.1. Plant material

In our experiments the following plant material was used: wheat cultivars ('Asakaze', 'Chinese Spring', 'Mv Bodri', 'Mv Karizma'), Mv9kr1 and Nannong 02Y23 wheat lines; barley cultivars ('Manas', 'Betzes'); wheatgrass species: *Pseudoroegneria spicata* (St genome), *Thinopyrum bessarabicum* (J<sup>b</sup> genome), *Thinopyrum elongatum* (E genome), *Thinopyrum intermedium* (JJ<sup>b</sup>St); genetic materials: 'Asakaze'/'Manas' wheat/barley disomic addition lines (2H, 3H, 4H, 6H, 7H), (Molnár-láng et al., 2012), 'Chinese Spring'/'Betzes' wheat/barley 3HS.3HL spontaneous centric fusion lines; progeny lines of wheat/*Agropyron glael* hybrids.

### 2.2. Genomic *in situ* hybridisation:

High-concentrated DNA (>1000µg/mL) was isolated from the species carrying the genomes we aimed to detect. The H (*Hordeum vulgare*), J (*Th. bessarabicum*) and St (*Pseudoroegneria spicata*) genomes were labelled with biotin-11-dUTP or digoxigenin-11- dUTP molecules using random priming or nick-translation methods. GISH was carried out in the presence of the probe (or probes in the case of mcGISH) and blocking DNA (the recipient genome), at

42°C. The detection of the labelled sequences was carried out using Streptavidin-FITC or Antidig-Rhodamine antibodies. Detection of the fluorescent signs was made using the method described by Kruppa et al. (2012).

### **2.3. Fluorescent *in situ* hybridisation:**

The used probe combinations were the following: Afa family (red), pSc119.2 (green), pTa71( yellow) for the identification of wheat chromosomes; (AGGGAG)<sub>4</sub> (red), (GAA)<sub>7</sub> (green), HvT01(yellow) for the identification of barley chromosomes; the pre-treatments and detection steps with small changes were the same as those described for GISH; the hybridization was carried out at 37°C, the post hybridisation wash made using 4×SSC (37°C, 2×5 minutes). The sequences labelled with biotin or digoxigenin, or their mixture were detected using Streptavidin-FITC or Antidig-Rhodamine antibodies solved in 10µg/mL TNB.

### **2.4. Plant growing and crosses**

Depending on genotype, the plants required 6-8 weeks of vernalisation after the plant growth in phytotron growth chambers using the method described by Tischner et al. (1997). The harvested plants were thoroughly examined (in terms of plant height, tillering, length of main spike, number of seeds/plant and number of seeds/spike) and the seeds were stored in gene bank container, at 4°C. The parameters of 10 plants/genotype were recorded.

The plants carrying the 3HS.3BL/CS/Mv9kr1 spontaneously developed centric fusion line were grown in 2009 in the ‘Tükrös’ nursery and the spikes of this line were emasculated and pollinated after 3-5 days with ‘Mv Bodri’ wheat cultivar using rotation technique. The seeds of the self-fertilised plants were screened for the presence of the homozygous centric fusion; the disomic plants were selected and grown in phytotron growth chambers. The descendants were sown in experimental plots and their agronomic traits were specified.

The descendants of Mv9kr1/*Agropyron glael* hybrid crossed with ‘Chinese Spring’ wheat cultivar were crossed with ‘Mv Karizma’, a facultative Martonvásár wheat cultivar (combination Mvkr1/*A. glael*//CS/3/Mv Karizma/4/Mv Karizma). Wheat/*A. glael* translocation lines were selected from the F<sub>2</sub> plants and were grown in phytotron growth chambers and experimental plots.

### **2.5. Salt stress resistance analysis of ‘Asakaze’/‘Manas’ wheat/barley ditelosomic addition lines**

The salt stress analysis was carried out in the ‘Asakaze’/‘Manas’ ditelosomic addition lines and parental (wheat and barley) genotypes at seedling stage. In the germination experiments the germinating seeds were treated with 0, 100, 200, 250 mM NaCl solution for 3 days and then the percentage of germinated seeds, the length and weight of the seedlings were determined.

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## **3. Results**

### **3.1. Identification and characterisation of ‘Asakaze’/‘Manas’ wheat/barley ditelosomic addition lines**

During the development of the ‘Asakaze’/‘Manas’ wheat/barley ditelosomic addition lines, the presence of the barley chromosome segments were detected by GISH, then SSR and STS marker analysis were used for their precise identification. A total number of 860 plants were analysed among the progenies of wheat/barley hybrid descendants. The following wheat/barley ditelosomic addition lines were selected: 2HS, 2HL, 3HS, 3HL, 4HS, 4HL, 6HS, 6HL, 7HS, 7HL. After multiplying the lines, the effect of the presence of certain barley chromosome arms was analysed (Türkösi et al., 2016). The 4HL ditelosomic addition line has the shortest plants and it shows the highest productivity of all

the developed genetic material. The line carrying the 7HL chromosome arm flowers earlier than the wheat control, while the lines carrying the 6H chromosomes or one of these chromosome arms flower the latest. The salt stress analysis of the lines showed higher salt stress tolerance of the 7HL line during germination and in the early developmental stages compared with the wheat parental genotypes (Darko et al., 2015). After multiplying the lines, their stability was investigated. The presence of the barley telocentric chromosomes was analysed among the descendants of the ditelosomic addition lines. The developed stability of the genetic material by each line was above 50%.

### **3.2. Introgression of the spontaneous 3HS.3BL centric fusion into 'Mv Bodri' wheat cultivar**

The earlier developed spontaneous 3HS.3BL translocation line maintained in small-plot experiments (CS/B//Mv9kr1/3/Mv9kr1) was crossed with winter wheat cultivar 'Mv Bodri' (CS/B//Mv9kr1/3/Mv9kr1/4/Mv Bodri) in order to improve the agronomic traits of the initial translocation line. The crossed F<sub>1</sub> plants were self-pollinated and the F<sub>2</sub> plants were screened for the presence of the homozygous centric fusion. The descendants of the plants carrying the disomic centric fusion were raised on experimental plots in Martonvásár nursery. These plants showed two phenotypes: one possessed awnless spikes, the other had spikes with awnstubs. The presence of *RhtD1b* dwarfing allele, which was previously mapped on the short arm of 4D of 'Mv Bodri' cultivar, was examined. The newly developed translocation lines were significantly shorter than the initial centric fusion line because of the introgression of the *RhtD1b* dwarfing allele after the cross with 'Mv Bodri' cultivar. The spikes of the awnless phenotype were similar in length to those of 'Chinese Spring', while the spikes of the phenotype with awnstubs were similar to those of 'Mv Bodri', but the fertility of the two lines were similar to each other despite the differences



in length. The introgression of 3HS.3BL compensating translocation in the genetic background of 'Mv Bodri' wheat cultivar has advantageous influence on the tillering and the number of seeds/plant (Türkösi et al., 2014).

### **3.3. Analysis of the Mv9kr1/A. *glael* terminal translocation (6DL.6DS-St)**

Leaf rust-resistant lines were selected from the descendants of Mv9kr1/A.*glael* hybrid that was crossed with wheat and self-pollinated. In order to reduce the number of *Thinopyrum* chromosomes, crosses with Mv9kr1 wheat line and 'Mv Karizma' cultivar were carried out. The plants were screened for their resistance against fungal diseases and the resistant plants were grown. Using mcGISH method wheat/*Thinopyrum* addition, substitution and translocation lines were identified. Their chromosome numbers were between 42 and 52. From the Mv9kr1/A. *glael*//CS/3/ Mv Karizma/4/Mv Karizma plants a genotype containing terminal translocation was selected. The identification of the chromosome involved in the translocation was carried out using specific repetitive DNA probes (pSc119.2, Afa family and pTa71) and the translocated chromosome was identified as 6DL.6DS-?St terminal translocation. Two plants carrying the homozygous translocation were selected from the F<sub>3</sub> generation using mcGISH. The inheritance of the translocation was detected by self-fertilising the plants: 19 homozygous plants were found among 40 analysed individuals. The presence of the Y38SCAR<sub>982</sub> telomere-specific marker linked to *Lr38* leaf-resistance gene was detected in the translocation line. In order to confirm the leaf rust resistance of the genetic material, artificial leaf rust inoculation needs to be carried out. The translocation lines were grown in the nursery in 2016/2017, where the plants showed no sign of leaf rust infection. However, in this period, even the susceptible borders were infected only mildly by leaf rust, which makes further investigations necessary. The spontaneous infection of the plants with powdery mildew justifies the molecular assays of

powdery mildew infection in the translocation line. The presence of two powdery mildew resistance genes (*Pm21* and *Pm962*) was followed up using molecular marker techniques. The receptiveness of the wheat parental genotypes denotes by all means the *Thinopyrum* origin of the powdery mildew resistance. Our experiment results prove unequivocally that genetic material originating from Mv9kr1/*A.glael* hybrid is a valuable genetic material for wheat improvement.

### **3.4. New scientific results**

1. A wheat/barley ditelosomic addition set (2HS, 2HL, 3HS, 3HL, 4HS, 4HL, 6HS, 6HL, 7HS, 7HL) was developed from 'Asakaze'/'Manas' disomic addition lines.
2. The line carrying the long arm of 7H chromosome (7HL) flowered the earliest from all ditelosomic addition lines and the wheat parental genotypes and lines carrying the 6H, 6HS and 6HL chromosomes flowered the latest.
3. The salt tolerance experiments confirmed the better salt tolerance of 7HL ditelosomic addition lines in comparison with the wheat parental genotypes and the other ditelosomic addition lines.
4. By crossing 3HS.3BL spontaneous translocation with 'Mv Bodri' winter wheat cultivar, a new genetic variability was developed and the agronomic traits of the translocation line were improved in comparison with the initial translocation line. We selected those individuals that carry both the *RhtD1b* dwarfing allele originating from 'Mv Bodri' cultivar and the 3HS.3BL Robertsonian translocation. The presence of 3HS chromosome arm had a

positive influence on tillering and balanced the absence of the 3BS chromosome arm.

5. The amount of *Thinopyrum* chromatine was reduced by crossing the Mv9kr1/A. *glael* hybrid with different wheat cultivars. From 269 progenies of these plants addition, substitution and translocation lines were selected. A terminal translocation carrying 42 chromosomes was selected and identified as 6DL.6DS-?St translocation line.

6. The molecular analysis of the 6DL.6DS-?St translocation line confirmed the presence of the Y38SCAR<sub>982</sub> marker and the telomere origin of the *Thinopyrum* segment.

#### **4. Conclusions and Recommendations**

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##### **4.1. Wheat/barley ditelosomic addition lines**

For the maintenance of wheat/barley ditelosomic addition lines – being aneuploids –, constant cytogenetic control is needed. The addition of barley chromosome segments to the hexaploid wheat genome makes gene mapping and describing the function of the genes localised on certain chromosome arm possible. The cytogenetically tested lines can be used for further molecular biologic and genomic analysis. The ditelosomic addition lines represents an opportunity to develop stable, euploid translocation lines that carry 42 chromosomes and that can be used effectively in pre-breeding programmes. In connection with this, the first aim is to develop compensating translocations, where a homeologous chromosome segment from the alien species can balance the absent wheat chromosome segment. The developed genetic material can be used for wheat breeding.

#### **4.2. The 3HS.3BL/Mv Bodri wheat/barley translocation lines**

For the wheat × barley crosses we used easily crossable wheat genotypes to increase the effectiveness of the crosses. The agronomic traits of the easily crossable wheat cultivars are weaker than those of the best, modern wheat cultivars. Our experimental results confirm that the wheat genetic background considerably influences the agronomic traits of the wheat/alien translocation line. Replacing the wheat genome with the genome of a modern, productive, resistant cultivar highly improves the agronomic traits of the developed genotypes. The presence of the 3HS chromosome arm improves tillering ability, which results higher seed number and balances well the absence of 3BS wheat chromosome arm. In the future, it would be advisable to carry out quality analysis on the genotypes as some vital amino-acids (e.g. lysine) are present in higher amount in barley. After manifold backcrosses, selection and multiplication, the lines can be used in breeding programmes.

#### **4.3. Wheat/*Thinopyrum* introgression lines**

Crosses between wheat and *A. glael* (*Th. intermedium* × *Th. ponticum* syntetic interspecific hybrid) make the transfer of resistance genes originating from *Thinopyrum* possible into cultivated wheat. The self-pollinated and crossed progenies of the hybrid developed in Martonvásár were maintained in our nursery; at the same time, the resistance of the lines was examined. The progenies of the resistant plants were screened using molecular cytogenetic methods (mcGISH, FISH) and addition, substitution and translocation lines were selected from them. The line carrying the *Thinopyrum* chromosome segment was identified as a 6DL.6DS-?St translocation line. The precise identification of the translocation line needs further cytogenetic, molecular and genomic analysis. To identify the breakpoint of the 6D chromosome, it is advisable to design new 6DS-specific molecular markers. The translocation line was originating from a leaf rust-resistant parent, so recording of spontaneous infection and artificial

inoculation of the lines should be carried out. In the case of leaf rust resistance, molecular analysis can reveal the molecular background of the resistance. In the phytotron growth chambers, the wheat parental lines became infected with powdery mildew, which makes the analysis of the powdery mildew resistance necessary in the developed introgression lines. The applied molecular marker analysis excluded the presence of *Pm21* and *PmL962* resistance genes in the analysed genotypes. Artificial inoculation of the lines with powdery mildew would be necessary in order to identify the molecular background of the resistance. Further crosses with ‘Mv Bodri’ cultivar would make the wheat background more balanced. The high abiotic stress tolerance of the *Thinopyrum* species (salt and drought tolerance) makes the examination of these traits necessary in the introgression line. In order to exploit the genetic diversity of the *Thinopyrum* species in wheat breeding programmes, it would be useful to collect these species, store them in gene bank and use them in crossbreeding programs.

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## 5. Publications

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