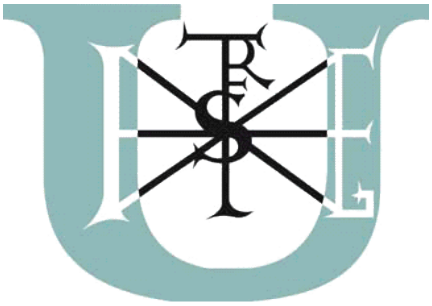

Szent István University

Development and characterization of *Triticum timopheevii* derived new genetic materials

Main points of the PhD thesis

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1 BACKGROUND AND AIMS

Bread wheat (*Triticum aestivum* L.) is one of the most important cereals of humanity, therefore essential work of wheat breeders is to continously increase its yield and yield safety. One effective way is the utilization of wheat wild relatives in breeding for resistance. One of these wild species is *Triticum timopheevii* Zhuk., which has outstanding resistance to the main wheat fungal pathogens (leaf-, stripe- and stem rust, powdery mildew, *Fusarium*). Beside direct interspecific hybridization, useful traits could be also successfully transferred into wheat through the development and utilization of a synthetic amphiploid using as a bridge for the gene transfer during the hybridization.The tetraploid *T. timopheevii* should be crossed with diploid einkorn (*Triticum monococcum* L.) to obtain an amfiploid having the same chromosome number as the hexaploid bread wheat and to utilize the outstanding biotic and abiotic resistance of einkorn as well. Amount of alien chromatin in the hybrids could be effectively decreased performing back-crosses (BC) in the offspring generations. During this prebreeding procedure, alien chromatin could be effectively identified using DNA (dezoxyribonucleic acid) *in situ* hybridization, where well known repetitive DNA sequences (FISH: fluorescent *in situ* hybridization) or total genomic DNA of the targeted genom (GISH: genomic *in situ* hybridization) are used as fluorescent probes.

Aim of our research is the utilization of the useful traits of the wheat wild relative, *T. timopheevii* in wheat breeding, which was planned to be achieved with the elaboration of the following tasks:

- Characterization of *T. timopheevii* accessions maintained in the Martonvásár Cereal Genebank of the Centre for Agricultural Research, Hungarian Academy of Sciences (MTA ATK) to select one as the initial plant material of a wheat prebreeding program with multiple direction, where resistance of hybrids to the main wheat fungal pathogens will be also investigated,
- Development and detailed morphologic, agronomic and molecular cytogenetic characterization of a new *T. timopheevii* × *T. monococcum* amphiploid using this selected *T. timopheevii* genotype and an einkorn line bred earlier in Martonvásár at the Agricultural Institute of MTA ATK, and its introduction into wheat prebreeding program,
- Continue the *T. timopheevii*-based prebreeding program at Martonvásár, which had preliminary resulted in the development of wheat addition lines carrying alien chromosomes,
- Improvement and optimization of molecular cytogenetic methods (FISH, GISH) to identify chromosomes and chromosome segments of *T. timopheevii* and *T. monococcum* introgressed in wheat during the prebreeding procedure. Development of FISH karyotype of *T. timopheevii* to ease the identification of alien chromosomes.

2 MATERIALS AND METHODS

2.1 Plant materials and crossing combinations

One (*T. timopheevii* subsp. *timopheevii* var. *rubiginosum* (MVGB845)) from the 56 *T. timopheevii* genebank accessions (MVGB) was selected for further prebreeding works.

A semi-dwarf einkorn line (*T. monococcum* subsp. *monococcum* '1T-1') bred at Martonvásár was used as male parent in the development of the amphiploid (*T. timococcum* Kost. nom. nud.).

As wheat genotypes, *T. aestivum* 'Mv9kr1' line carrying the recessive allele-pair *kr1kr1* – and thus having good crossability in interspecific hybridizations – and common wheat varieties ('Mv Karizma', 'Mv Marsall', 'Mv Nádor', 'Mv Suba') were used.

Controls used for the examinations of disease resistance of *T. timococcum* and its parents:

- Leaf rust: 'Mv9kr1' (susceptible), *T. aestivum* 'Alcedo' (susceptible), szülők (resistant),
- Powdery mildew: susceptible ('Mv9kr1', 'Carstens V.') and resistant ('Nannong 02Y23') wheat lines, *T. monococcum* 'Mv Alkor' (resistant), *T. zhukovskyi* MVGB650 (susceptible),
- Fusarium head blight: susceptible ('Mv222-13') and moderately resistant ('Mv213-11') wheats.

Wheat lines carrying *T. timopheevii* chromosomes bred earlier at Martonvásár were examined, including a *T. aestivum* × *T. timopheevii* 6G disomic addition line.

Total genomic DNA of *Triticum urartu* – MVGB115 (A) and *Aegilops speltoides* – MVGB905 (genome S: the ancestor of genomes B and G) were used in GISH.

Crossing combinations:

- *T. timopheevii* MVGB845 × *T. monococcum* '1T-1', named *Triticum timococcum*. The further generations of the amphiploid are referred to as C_n, instead of F_n,
- Further *T. timopheevii* × *T. monococcum* '1T-1' hybrids originated from test-cross trial,
- 'Mv9kr1' × *T. timococcum* and its BC_1, BC_2, BC_3 generations based on 'Mv9kr1',
- *T. timococcum* × wheat genotypes ('Mv9kr1', 'Mv Marsall', 'Mv Nádor', 'Mv Suba'),
- *T. zhukovskyi* MVGB650 × *T. timococcum*,
- 'Mv9kr1' × *T. timopheevii* MVGB845 and its BC_1 and BC_2 generations,
- *T. timopheevii* MVGB845 × wheat genotypes ('Mv9kr1', 'Mv Karizma', 'Mv Marsall', 'Mv Nádor', 'Mv Suba'),
- *T. aestivum* 'Rannaja' 6B monosomic line MVGS1117 × *T. aestivum* 6G disomic addition.

2.2 Development of interspecific hybrids

Spikes of the female parents of the crossing combinations were emasculated and pollinated 2-4 days later using spikes cut from male parents. Regeneration and crossings of plants were carried out in field and in phytotron as well.

Seed set was determined after harvest using the ratio of seeds developed on a spike and the number of flowers pollinated on a spike.

The triploid genome of the *T. timopheevii* × *T. monococcum* F_1 hybrid was doubled by soaking the roots of plants in 3-5 leaves growth stage in 0.04% colhicine solution.

2.3 Molecular cytogenetic analysis

2.3.1 Making and pretreatment of chromosome preparations

Root tips cut from the germinated seeds were fixed in ice-cold water followed by fixation in 3:1 solution of absolute ethanol and acetic acid and than staining in acetocarmine. Mitotic chromosome preparations made from these root tips were examined with phase contrast microscope followed by storage at –20 °C until utilization in *in situ* hybridization.

Before fluorescent *in situ* hybridization (FISH és GISH) RNA and cytoplasm were removed from the preparations using ribonuclein enzyme and pepsin, respectively, followed by a rinsing procedure and a fixation with paraformaldehyde solution. Preparations were dehydrated with ice-cold ethanol series (3-3 minutes 70% and 90%, 5 minutes 100% ethanol). In the case of GISH performed after FISH, preparations were washed in 4× SSC-Tween before the rehybridization.

2.3.2 Fluorescent *in situ* hybridization

DNA probes used in the fluorescent *in situ* hybridization were labelled with digoxigenin-11-dUTP and/or biotin-16-dUTP by nick translation in order to detect their hybridisation spots on the chromosomes of interest through the help of fluorochromes bound to them.

Master mix (~28 µl) of the hybridization mixture (30 µl/slide) contained formamide, 25 v/v% dextran sulphate, 20× SSC and 10 v/v% SDS in a ratio of 50:33:10:1. Blocking DNAs were added to this mixture in the form of 5 ng (density: 50 ng/µl) salmon sperm DNA (FISH) or 2 µg (50 times the quantity of the labelled S genomic probe) S genomic DNA (GISH).

Following quantities of labelled probes were added to the master mix:

- FISH: 20 ng (0.4 µl) Afa-family (digoxigenin), 30 ng (0.6 µl) pSc119.2 (biotin) és 30 ng (0.6 µl) pTa71 (biotin and digoxigenin in 1:1 ratio)
- GISH: 40 ng (0.8 µl) A genomic probe labelled with biotin and 40 ng (0.8 µl) S genomic probe labelled with digoxigenin

DNAs in the hybridization mixture were denaturated on 85 °C for 12 minutes (FISH) or on 99 °C for 10 minutes (GISH) using PCR followed by the denaturation of the chromosomes on the slides (in the presence of the denaturated mixture) on 75 °C for 6 minutes (FISH) or on 80 °C for 2 minutes (GISH). After denaturation, preparations were incubated for 16 hours on 37 °C (FISH) or 42 °C (GISH), than a stringency washing with SSC series were carried out on the slides.

During a hybridization process on 37 °C for 20 minutes (50 µl/slide), the hybridization spots of the probes labelled with digoxigenin and biotin were labelled with red fluorescent anti-digoxigenin-rhodamine Fab (antigen-binding) fragments and green fluorescent streptavidin-FITC Fab fragments, respectively. After a washing procedure in 4× SSC-Tween counterstaining of the preparations was carried out using 2 mg/ml DAPI/Vecta Shield solution (20 µl/slide). The slides were examined with Zeiss AxioImager.M2 fluorescence microscope, documented with Zeiss AxioCam MRm CCD camera and evaluated with AxioVision 4.8.2 software.

2.4 Resistance analysis of seedlings

2.4.1 Artificial leaf rust inoculation

Optimum cicumstances for leaf rust inoculation carried out in greenhouse were ensured with polyethylene cover during the first 2 days. On the 10th day after the inoculation of seedlings having 2 leaves, severity of disease symptoms were evaluated using a six-grade scale (0, ; , 1-4).

2.4.2 Artificial powdery mildew inoculation

Inoculation was carried out in isolated glass boxes on the 6th day after sowing using conidia of powdery mildew races 51 and 76 followed by the evaluation (0-4 scale) on the 7th day thereafter. Besides, interactions between pathogen and host were also examined on different genotypes using leaf preparations whitened with acetic acid and stained with aniline blue and DAB. Preparations made 9 times (every 8th hours after inoculation, than on the 3rd, 4th and 7th days) and examined with Zeiss AxioScope.A1 microscope were documented with Canon digital camera.

2.5 Resistance analysis against *Fusarium* head blight

Resistance to scab (isolates of *F. graminearum* ‘IFA-66’ and *F. culmorum* ‘IFA-104’) was determined (%) in two ways, on the 26th day after spraying (field resistance) and on the 21st day after inoculation of spikelets with the pathogen. During the latter procedure (type II resistance) 5 spikes from each genotypes were inoculated and their results were also evaluated statistically.

2.6 Phenotypic description

Growth habit, growth form, heading date, plant height and thousand kernel weight of the genotypes were determined under field conditions. Their resistance to the main wheat fungal diseases (powdery mildew, leaf rust, stripe rust) was also assessed under natural pathogen pressure using 0-9 scale for scoring (0 = resistant, 9 = susceptible). In parallel with the field trials, the amphiploid and its parents were examined also in phytotron in years 2012 and 2013, where spike morpholgic traits were also assessed. Assessment datasets were analysed statistically (ANOVA and post hoc test) as well.

3 RESULTS AND DISCUSSION

3.1 Development of *Triticum timococcum*

3.1.1 Characterization and test-cross of *Triticum timopheevii* genebank accessions

Altogether 56 *T. timopheevii* accessions maintained in the Martonvásár Cereal Genebank were characterised. Based on our results, it was concluded that the female parent of the new amphiploid should be selected from the group of 38 subsp. *timopheevii* accessions as they have adequate agronomic value. Despite the advantageous early heading date of subsp. *armeniacum* accessions, their fragile stachis and relatively small seeds could make their utilization in wheat prebreeding less effective.

For the test-crosses with 11 selected early heading subsp. *timopheevii* accessions under field conditions, a semi-dwarf einkorn line (’1T-1’) was used which was previously selected as male parent of the amphiploid. The genebank accession (*Triticum timopheevii* Zhuk. subsp. *timopheevii* var. *rubiginosum* (MVGB845)) with the highest seed set (16%) having relatively good agronomic value was selected not only for the development of the amphiploid, but also for direct crossing with wheat.

3.1.2 Development of *Triticum timopheevii* × *Triticum monococcum* amphiploid

Altogether 255 F_1 hybrid seeds were developed after crossing the selected *T. timopheevii* MVGB845 accession (based on the results of the assessment and test-cross experiments) with the semi-dwarf einkorn line, ’1T-1’. Triploid plants developed from these seeds were doubled with colhicine resulted in a fertile hexaploid generation (C_1) named *Triticum timococcum.*

Botanical name of the *T. timopheevii* × *T. monococcum* amphiploid derived from the species denominations of the parents is officially not accepted yet, therefore this synthetic species should be named *Triticum timococcum* Kost., nom. nud. at the first appearance in scientific publications. The hybrid-development was found to be more effective regarding seed set than in other research works dealing with *T. timococcum*. Our findings also reinforce the fact that plant materials can only be utilized effectively in the development of synthetic amphiploids with good agronomic value after an appropriate selection and prebreeding process.

3.2 Molecular cytogenetic identification of *Triticum timococcum*

3.2.1 Karyotyping and GISH optimization of the parental genomes

As a first step in the identification of the amphiploid’s genome, FISH karyotypes of the parental genomes were developed using DNA probes (Afa-family, pTa71 and pSc119.2) widespread in

wheat research resulted in more effective identification of all the *T. timococcum* chromosomes during the forthcoming works (Figure 1).

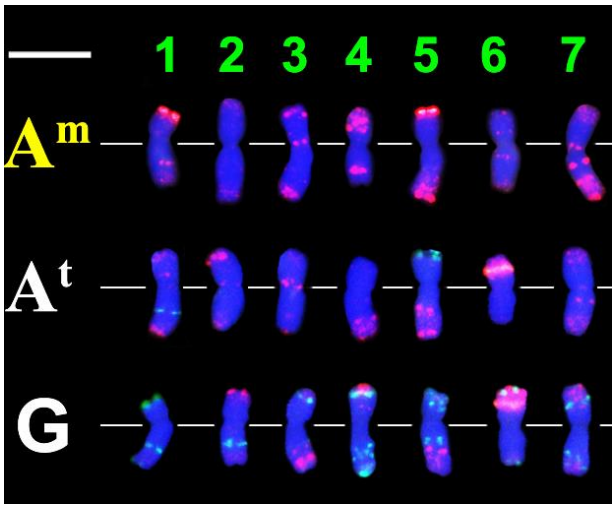

Figure 1 FISH Karyotypes of A^m, A^t and G genomes: fluorescence *in situ* hybridization patterns of repetitive DNA probes pSc 119.2 (green), Afa-family (red) and pTa71 (orange) on chromosomes of *Triticum monococcum* subsp. *monococcum* '1T-1' (A^m) and *Triticum timopheevii* subsp. *timopheevii* var. *rubiginosum* MVGB845 (A^t and G) arranged according to genomes and homeologous groups (Bar = 10 μm)

The different evolution of tetraploid *Triticum* species carrying G or B genome is supported also by the abscence of pSc119.2 patterns on the $4A^tL$ chromosome arm of *T. timopheevii* that are present on the 4A chromosome of *T. turgidum* (and *T. aestivum*). This missing fragment could be translocated on the terminal region of chromosome arm 4GS during the translocation $4A^tL/4GS$, which translocation was also resulted in the appearance of strong Afa-family FISH signs on the terminal region of 4GS. All the G chromosomes can be precisely distinguished from each other and from the B chromosomes based on their FISH patterns.

Discrimination of the G and A genomes of the amphiploid was carried out with multicolour GISH (mcGISH) supported by an optimization procedure carried out earlier on the *T. timopheevii* MVGB845 accession. Quantity and ratio of probe and blocking DNAs were adjusted resulted in parameters detailed in 2.3.2. Beside the discrimination of G and A chromosomes, species-specific intergenomic translocation ($T6A^tS/1GS$) of *T. timopheevii* was also identified.

3.2.2 Genome composition of *Triticum timococcum*

With consecutive using of the optimized FISH and mcGISH techniques, the 42 chromosomes of the amphiploid were identified as the derivatives of all parental chromosomes and the species-specific intergenomic translocation of *T. timopheevii* was also found in the amphiploid. On the basis of our results, the genome composition of the newly developed synthetic amphiploid, *Triticum timococcum* can be described as 2n=6x=42, $A^tA^tGGA^mA^m$.

Our results help to increase the effectiveness of the identification of *T. timopheevii* chromosomes using fluorescent *in situ* hybridization techniques (FISH, mcGISH). Furthermore, our study was the first that could prove the effective utilization of these optimized techniques in the examination of the *T. timococcum* genome.

Based on the specific FISH patterns, most A^m chromosomes of the ancient diploid species, *T. monococcum* could be clearly distinguished from A^t chromosomes and A^u chromosomes of wheat in the amphiploid and in its hybrids with wheat during prebreeding. In contrast, only $1A^t$, $3A^t$, $4A^t$ and $6A^t$ chromosomes of *T. timopheevii* could be distinguished from homeologus wheat chromosomes, because of the translocations occured during its evolution.

Based on our results, chromatins of *T. timococcum* and *T. timopheevii* introgressed into wheat could be effectively identified during the prebreeding program in the offspring generations using FISH and mcGISH.

3.3 Phenotypic and agronomic characterization of *Triticum timococcum*

3.3.1 Morphologic characteristics

Strong significant differences were found between the amphiploid and its parents for most of the traits examined in the field for two years. In contrast, the comparison in the phytotron showed significant differences for fewer traits. Unlike the results of the field studies (80 cm), the plant height of *T. timococcum* (100 cm) showed a greater resemblance to that of *T. timopheevii* in the phytotron. Average heading date of the autumn sown amphiploid was in the middle of June. Awned spikes and spikelets of the amphiploid were intermediate to that of the parents, and it developed longer and looser spikes than its parents, which was manifested even more in the later generations due to the targeted selection (Figure 2).

Figure 2 Spikes of *Triticum timopheevii* MVGB845 (left), *T. monococcum* '1T-1' (middle) and *T. timococcum* C_5 generation (right) gathered from field (Martonvásár, 2015; Bar = 1 cm)

T. timococcum has facultative growth habit, like its parents, and being more pubescent than the *T. timopheevii* parent, which could be advantageous against virus vector insects and drought. Compared to its parents, the amphiploid with doubled genome has the longest stoma (100 μm), which was examined with light microscope.

3.3.2 Disease resistance

Young and adult plant resistance of the amphiploid to leaf rust and its adult plant resistance to stripe rust (score: 0) are derived from the effective resistance genes carried by both of its parents. However, information on the susceptibility of *T. timopheevii* and *T. monococcum* seedlings to powdery mildew could not be found in the literature. These species and the amphiploid derived

from them could also have genes expressing adult plant resistance, which could be explained by the slow development (having atypic germination on einkorn) of powdery mildew infection (under high pathogen pressure) on the young plants and by the resistance documented on adult plants in the field. Infection resulted in hypersensitive reactions on the *T. zhukovskyi* and *T. timopheevii* seedlings. Moreover, resistant individuals were selected from the populations of the *T. timopheevii* accession and the amphiploid.

During the examination of the powdery mildew – host plant interaction, development and spread of the pathogen (aniline blue) and the occurent defensive response of genotypes (accumulation of hydrogen peroxide (H_2O_2) stained with DAB, papillae development) were analysed. Compatible pathogen – host interaction was found on five genotypes showing different progressions of the disease, while the genotypes 'Mv Alkor' and 'Nannong 02Y23' were resistant. Despite *T. timococcum* was susceptible to powdery mildew, it responded to the primary germination tube of the conidium with local accumulation of hydrogen peroxide, while other genotypes did not show such early reaction. Later on, whole cells of einkorn showed DAB staining, which could also refer to the moderately resistance found on this genotype. Sporulation of powdery mildew was documented on the susceptible wheat variety on the 4^{th} day after inoculation, while this occured on the amphiploid and its parents only on the 7^{th} day.

As a result of the *Fusarium* inoculation carried out on adult plants, the *T. timopheevii* genotype (MVGB845) was found to be moderately resistant (*F. graminearum*: 5%; *F. culmorum*: 25%; type II resistance: 12% for both isolates), while the semi-dwarf einkorn genotype ('1T-1') was 100% infected. Based on this, field resistance (*F. graminearum*: 5%; *F. culmorum*: 15%) and type II resistance (*F. graminearum*: 15%; *F. culmorum*: 5%) of the amphiploid developed from these two species are inherited from the female parent. In the case of both *Fusarium* isolates, the amphiploid showed better field resistance than the moderately resistant wheat control ('Mv213-11'), albeit this difference was not significant by the evaluation of type II resistance.

3.4 Development of wheat prebreeding material carrying *Triticum timopheevii* chromatin

3.4.1 Crosses with the amphiploid, *Triticum timococcum*

In the summer of 2012 a prebreeding program was started based on the utilization of the amfiploid, where the wheat line 'Mv9kr1' was used as main crossing partner. According to the results of the development of F_1 hybrids in both crossing directions, seed set could be three times higher if the wheat genotype is used as female parent (19.5%).

During back-crosses with the genotype 'Mv9kr1', the lowest seed set was obtained after the first back-cross, even lower when the amphiploid was used as female parent in the F_1 hybrid (seed set: 0.64%). Back-cross of the F_1 hybrids having wheat cytoplasm was much more effective. In

this case, the first back-cross was also resulted in low seed set, but it had increased by the third back-cross to more than 18%. BC_3 progenies originated from this prebreeding program were multiplied in greenhouse by the summer of 2015 for field trials planned in the near future.

As the amphiploid has the same genome as the natural species *T. zhukovskyi*, genetic diversity of the latter species was widen by crossing them. The genome composition of the *T. zhukovskyi* MVGB650 × *T. timococcum* F_3 generation was examined with mcGISH and the species-specific translocation of *T. timopheevii* was also identified in the hybrid. Seedlings of this hybrid were found to be resistant to artificial leaf rust infection.

3.4.1.1 Genome analysis of the offspring generations

According to the result of the cytogenetic analysis of F_1 hybrids originated from crosses between wheat and the amphiploid, 75% of the plants originated from both crossing directions had 42 chromosomes, which were used in further back-crosses.

Several G chromosomes of *T. timopheevii* and also one einkorn chromosome ($1A^m$) were identified by the FISH analysis of BC_1 plants having wheat cytoplasm. Chromosomes 1G, 2G and 6G were found most frequently (in all cases without their pair), where they mostly substituted one part of their homeologues B chromosme-pairs (monosomic substitution).

About 30% of BC_2 progenies had 42 chromosomes due to the negative effect of the relatively high ratio of alien chromatin on the process of normal cell division. Like in the previous generation, monosomic substitutions of G chromosomes were identified, but there were less alien chromosomes in an average plant compared to the BC_1 plants. As a result of the third back-cross, we assume that translocations will occure in the monosomic substitutions between the B and G chromosomes homeologous to each other.

3.4.1.2 Phenotypic description and artificial leaf rust inoculation of the offspring generations

Introgression of alien chromatin was indicated by variable spike shape in the consequtive generations, where ’Mv9kr1’-like awnless spikes were also found in the BC_3 generation. Average plant height of the F_1 hybrids (100 cm) was almost halved due to back-crosses with wheat. F_1 hybrids having *T. timococcum* cytoplasm developed abnormal spikes with decreased fertility and their plant height was under 40 cm.

Seedlings of BC_2 generation were artificially inoculated with leaf rust resulted in immunity (score: 0) by more than half of the plants and in different level of resistance (score: 1 or 2) by 25% of the plants that were used in further crossings.

3.4.2 Crossings with *Triticum timopheevii*

Beside the development, examination and utilization of *T. timococcum*, prebreeding program was started also with the earlier selected *T. timopheevii* genebank accession (MVGB845) in the winter of 2012/2013. During this direct gene transfer of *T. timopheevii*, hybrids were made with 'Mv9kr1' wheat line followed by back-crosses with 'Mv9kr1'. The relatively high seed set (40%) of the F_1 hybrid-development was greatly decreased (5%) by the first back-cross (BC_1), but large number of progenies were obtained again in the BC_2 generation. These plants were multiplied in greenhouse by the summer of 2015 for field trials planned in the near future.

3.4.2.1 Genome analysis of the back-crossed progenies

FISH analysis was first carried out on the BC_1 generation, as F_1 progenies of the hexaploid wheat and the tetraploid *T. timopheevii* had relatively unstable genome, resulted in the identification 42 chromosomes in 30% of the progenies examined. These plants were used in further back-crosses. Chromosome 1G was identified most frequently. Furthermore, chromosomes 2G, 6G and $5A^t$ were also found in the progenies that were mostly involved in monosomic substitutions, like chromosome 1G. Presumably, transloctaion of *T. timopheevii* chromatin will occure in the next generations.

3.4.2.2 Phenotypic description and artificial leaf rust inoculation of the offspring generations

Introgression of alien chromatin was indicated by variable spike shape in the offspring generations, where 'Mv9kr1'-like awnless spikes were also found in the BC_2 generation. Plant height of BC_2 plants (51 cm) was almost similar to that of wheat due to previous back-crosses.
Seedlings of BC_1 progenies purely based on crosses with 'Mv9kr1' and BC_1-like progenies having wheat varieties of Martonvásár in their pedigree were inoculated with leaf rust resulted in immunity or hypersensitive reaction (score: 0 or ;) by more than 66% of the plants that were used as female parents in further back-crossings.

3.4.2.3 Development of wheat lines having Triticum timopheevii cytoplasm

Beside the BC progenies having wheat cytoplasm, back-cross program with *T. timopheevii* × *T. aestivum* hybrids having cytoplasm of *T. timopheevii* MVGB845 was also started in order to develop cytoplasmic male sterile (CMS) wheat lines based on wheat varieties of Martonvásár.
Effectiveness of hybrid-development was three times higher when 'Mv9kr1' was used as pollinator (seed set: 20%) compared to the seed set (6.5%) obtained from pollinating with normal wheat varieties. Seed set of hybrids having *T. timopheevii* cytoplasm was realatively high, but the germination of F_1 seeds were very poor and the first back-crossing was also ineffective. This program will be continued with the remaining F_1 and F_2 seeds, expanding it with marker-assisted

selection (MAS) during the development of the male wheat breeding lines of the hybrid-combinations, which targets the fertility restoring genes.

3.4.3 Development of 6B/6G monosomic substitution wheat line (20^{II} +1^{I} 6B+1^{I} 6G)

Similar monosomic substitution could be induced, like those resulted preliminary from the back-cross programs described above, by crossing a wheat line carrying alien disomic addition with a monosomic wheat line. As a first step, after 10 years of storage, wheat genetic material developed earlier at Martonvásár and carrying the chromatin of the wild subspecies of *T. timopheevii* was multiplied, and put in artificial leaf rust inoculation trial in the beginning of 2013. Seedlings showing immunity to leaf rust were chosen for FISH analysis, where a line additionally carrying two 6G chromosomes (thus having 44 chromosomes) was identified (21^{II} + 1^{II} 6G). A *T. aestivum* 'Rannaja' 6B monosomic (20^{II} + 1^{I} 6B) wheat line (accession number: MVGS1117) developed earlier at Martonvásár was used as female parent in the crossing with this 6G disomic wheat line. F_1 progenies having 1 6B and 1 6G chromosomes among their 42 chromosomes (20^{II} +1^{I} 6B+1^{I} 6G) were identified with FISH and their multiplication was finished in autumn of 2015. Pairing of the 6B and 6G chromosomes, and thus translocations between them are expected in the F_2 generation.

3.5 New scientific results

1. Main phenotypic, agronomic traits and the crossability with einkorn of the *Triticum timopheevii* genebank accessions maintained at the Martonvásár Cereal Genebank were assessed and characterized. On the basis of our results, one accession (*Triticum timopheevii* subsp. *timopheevii* var. *rubiginosum* – MVGB845) was chosen that could be useful in wheat prebreeding.
2. A synthetic amphiploid, *Triticum timococcum* Kost., nom. nud. was developed from the crossing of this selected *T. timopheevii* accession (MVGB845) with a semi-dwarf einkorn line (*T. monococcum* subsp. *monococcum* '1T-1'). As a result of the crossability test, 9 other *T. timococcum* lines were also developed and multiplied in field, which is globally outstanding.
3. Phenotypic and resistance characters of *T. timococcum* and its parents were described in details. Susceptibility of *T. timopheevii* and *T. monococcum* seedlings to powdery mildew was found, therefore their resistance in the field could be ensured by adult plant resistance genes.

4. The fluorescent *in situ* hybridization (FISH) procedure was optimized for *T. timopheevii* chromosomes resulted in the development of FISH karyotypes of the genomes G and A^t using repetitive DNA probes pSc119.2, Afa-family and pTa71. These karyotypes were successfully applied at the genome analysis of the amphiploid and for the identification of alien chromatin in the hybrids of these species and wheat.
5. A multicolour genomic *in situ* hybridization (mcGISH) procedure was improved resulted in the clear discrimination of genome G from the two A genomes and also in the identification of the species-specific translocation of *T. timopheevii* in the amphiploid and the progenies. Identification of the chromosomes of the new amphiploid in the offspring generaions was made more effective by the consequtive application of the optimized FISH and mcGISH techniques.
6. Greater effectiveness of crossing *T. timopheevii* with bread wheat compared to the hybridization carried out on the hexaploid level using *T. timococcum* was confirmed. Outstanding crossability of 'Mv9kr1' wheat line carrying recessive crossability allele was also confirmed in comparison to common wheat cultivars.
7. Wheat prebreeding program was started based on *T. timopheevii* MVGB845 and *T. timococcum* using back-cross (BC) technique in order to exploit the outstanding diseasae resistance of these two species. During the BC program based on 'Mv9kr1', leaf rust resistant progenies was selected from the inoculated seedlings.
8. A 6G disomic addition wheat line was selected from the aneuploid wheat genetic material developed earlier at Martonvásár and carrying the chromatin of *T. timopheevii*, which was found to be resistant in the leaf rust inoculation trial carried out on seedlings. Plants carrying 6B/6G monosomic substitution were developed from the crossing of this 6G disomic addition line and *T. aestivum* 'Rannaja' 6B monosomic wheat line (MVGS1117), which result was proved with FISH.
9. *T. timopheevii* × *T. aestivum* and *T. timococcum* × *T. aestivum* hybrids were developed in order to have cytoplasmic male sterile wheat lines based on varieties of Martonvásár that would be a useful starting material of a *T. timopheevii* cytoplasm-based hybrid wheat breeding program.
10. Hybrids of *T. zhukovskyi* MVGB650 and *T. timococcum* were developed and characterized in order to widen the genetic diversity of the natural form of our amphiploid.

4 CONCLUSIONS AND RECOMMENDATIONS

4.1 Development of *Triticum timococcum*

Many useful information were gathered as a result of the assessment in several years of *T. timopheevii* acessions maintained in the Martonvásár Cereal Genebank, that could be used in targeted selection of the partners of interspecific hybridizations in the future. however, these phenotypic and resistance data have to be completed also with results of the responses to abiotic stresses. According to our results, it can be colcluded that there are some candidate *T. timopheevii* accessions beside the one (MVGB845) selected for the development of the amphiploid, that could be also introduced in wheat prebreeding programs to increase the genetic basis of breeding materails, even by means of the development of new *T. timococcum* lines.

Including the amphiploid examined in details in the present study, altogether 10 *T. timococcum* lines were developed from different *T. timopheevii* accessions. The fourth generation (C_4) of these other amphiploid lines are currently involved in a selection program under field conditions, and their progenies will be also introduced in wheat prebreeding programs in the near future.

A *T. zhukovskyi* × *T. timococcum* hybrid was developed to widen the genetic diversity of the natural species, *T. zhukovskyi*, which is currently in the fifth generation (F_5) and its multiplication will be continued.

The synthetic amphiploid, *Triticum timococcum* was also developed earlier, but always from the hybridiaztion of the wild subspecies of *T. timopheevii* and a natural type of einkorn. These parents also inherited disadvantageous traits (e.g. brittle rachis, poor straw strength) into their progenies. Our findings, however, reinforce the fact that plant materials can only be utilized effectively in the development of synthetic amphiploids with good agronomic value after an appropriate selection and prebreeding process.

4.2 Molecular cytogenetic identification of *Triticum timococcum*

During the present study, FISH karyotypes of the genomes of *T. timopheevii* (A^t and G) and *T. monococcum* (A^m) were created using repetitive DNA probes pSc119.2, pTa71 and Afa-family. Using the same probes, all the seven G, seven A^t and seven A^m chromosome pairs could be identified in the amphiploid based on their fluorescent *in situ* hybridization (FISH) patterns. Genome G of the hexaploid amphiploid could be clearly discriminated from its two A genomes with multicolour genomic *in situ* hybridization (mcGISH) optimized in the present study.

Based on our results, alien chromosomes of *T. timococcum* and *T. timopheevii* introgressed into wheat could be effectively identified during the prebreeding program in the wheat background of the offspring generations. The results of our karyotyping work could be used not only in wheat

prebreeding programs, but also in mapping and isolating genes, designing chromosome-specific markers and in the idenficiation of separated chromsomes resulted from sorting with flow cytometric technique.

4.3 Phenotypic and agronomic characterization of *Triticum timococcum*

Phenotype of the amphiploid was intermediate to that of the parents. Its dense pubescence is an advantageous morphologic trait, which could be also transferred into wheat to increase its defense against virus vector insects and drought. However, detailled examination of this topic is one of our future tasks.

Beside the outstanding resistance of *T. timococcum* to leaf- and stripe rusts, it was also resistant to powdery mildew, which is supposed to be due to genes regulating adult plant resistance, because seedlings of this amphiploid and its parents (both are recorded as resitant in the literature before) were sensitive to powdery mildew in our artifically inoculated trial. Because of the lack of research projects having similar subject, validation of this hypothesis through further experiments is an important task planned in the near future. Resistant individuals to powdery mildew were selected from the populations of the *T. timopheevii* accession and the amphiploid, that are currently under multiplication.

Hopefully, the relatively good resistance of *T. timococcum* to *Fusarium* head blight could be introgressed into wheat genotypes. This prebreeding work has already started and the examination of the repeatedly back-crossed progenies will be carried out in the near future.

According to the results of the stoma analysis, it was concluded that water use efficiency and drought tolerance of the amphiploid putatively do not differ from the parents that have good drought tolerance. However, this should be empirically tested in the future.

Yearly selection of *T. timococcum* shows promising results (productivity has increased with 50%), which should be continued in the future with the C_5 generation in order to have an agronomically acceptable, stable, homogenous plant material for the breeders.

4.4 Development of wheat prebreeding material carrying *Triticum timopheevii* chromatin

A wheat prebreeding program with multiple directions was started using back-cross (BC) technique to develop possibly the best performing hybrids carrying the useful genes of *T. timopheevii*. Additionally, a 6G disomic addition line was selected from the wheat genetic materials developed earlier at Martonvásár, which was used as a bridge material, like *T. timococcum*, and were crossed with a monosomic wheat line in order to develop a 6B/6G monosomic substitution line.

Also new einkorn derived genes could be introgressed into wheat by using *T. timococcum* in the prebreeding that could increase the disease resisatnce and quality parameters (tocol- and

carotenoid content) of wheat. According to our preliminary results, the accession *T. timopheevii* MVGB845 has outstanding α-tocopherol-, α-tocotrienol- and micronutrient (Se, Mn, K) content compared to wheat, therefore the detailed analysis and the introgression of these traits into wheat will be one of our most important tasks in the future.

Repeated back-crossing carried out during prebreeding was appeared to be an effective method for introgression of alien chromatin. However, this work will have final result only after some more years, when the wheat genotypes will carry only small part(s) of the alien chromosome with the useful gene(s) on it. Preliminary findings of this time consuming prebreeding procedure are promising, and the field tests of the BC generations planned in the future will give further useful results.

FISH karyotype of *T. timococcum* created in the present study was useful for the identification of alien chromatin in the BC progenies and could be recommended for the molecular cytogenetic evaluation carried out in other wheat breeding programs based on *T. timopheevii*.

Alien monosomic substitutions were detected in most of the progenies examined, therefore, due to further bock-crossings, an increasing number of translocations between the homeoelougous chromosomes involved in the monosomic substitutions are expected.

Task of the future is the improvement of the discrimination of B and G chromosomes, and also the A chromosomes with different evolutionary origin in order to identify the intergenomic translocations more effectively. Therefore, the selection of *T. timopheevii*-specific polimorph molecular markers is an important task for the future. The 6B-6G polimorph markers could be already used in the marker-assisted selection (MAS) of the progenies of the 6B/6G monosomic substitution lines that could putatively carry translocations. Using these markers, the origin, length and position of the translocated 6G chromosome segment(s) will be identified on the 6B chromosome of the progenies.

MAIN PUBLICATIONS OF THE AUTHOR

Scientific publications:

Publications in international scientific journals:

Mikó P., Megyeri M., Farkas A., Molnár I., Molnár-Láng M. (2015): Molecular cytogenetic identification and phenotypic description of a new synthetic amphiploid, *Triticum timococcum* ($A^tA^tGGA^mA^m$). *Genetic Resources and Crop Evolution*, 62(1): 55-66. p.

Mikó P., Löschenberger F., Hiltbrunner J., Aebi R., Megyeri M., Kovács G., Molnár-Láng M., Vida G., Rakszegi M. (2014): Comparison of bread wheat varieties with different breeding origin under organic and low input management. *Euphytica*, 199(1-2): 69-80. p.

Publications in Hungarian scientific journals:

Mikó P., Megyeri M., Molnár-Láng M., Kovács G. (2013): Characterization of *Triticum timopheevii* Zhuk. gene bank accessions for development of synthetic amphiploid wheat lines. *Acta Agronomica Hungarica*, 61(2): 113-121. p.

Megyeri M., **Mikó P.**, Molnár I., Kovács G. (2011): Development of synthetic amphiploids based on *Triticum turgidum* x *T. monococcum* crosses to improve the adaptability of cereals. *Acta Agronomica Hungarica*, 59(3): 267-274. p.

Other scientific publications:

Conference proceedings:

Mikó P., Löschenberger F., Megyeri M., Vida G., Rakszegi M., Molnár-Láng M. (2014): Responses of bread wheat varieties to different environments. *In: Chable et al. (eds.): Diversity strategies for organic and low input agricultures and their food systems. Book of abstracts of Solibam final congress, 7-9. July 2014.* SOLIBAM, Nantes, France, 35-36. p.

Megyeri M., **Mikó P.**, Molnár I., Taborská J., Pálfi X., Dulai S., Rapi S., Korózs M., Forgó P., Molnár-Láng M. (2014): Functional compounds of einkorn and emmer genotypes. *In: Chable et al. (eds.): Diversity strategies for organic and low input agricultures and their food systems. Book of abstracts of Solibam final congress, 7-9. July 2014.* SOLIBAM, Nantes, France, 96-97. p.

Mikó P., Megyeri M., Molnár-Láng M. (2014): Novel utilization of wild relatives in bread wheat prebreeding. *In: Kőszegi, I. (ed.): Advances in plant breeding and biotechnology techniques – book of abstracts, 27-29. April 2014.* Pannonian Plant Biotechnology Association, Mosonmagyaróvár, Hungary, 59-61. p.

Mikó P., Megyeri M., Molnár I., Molnár-Láng M. (2014): *Triticum timococcum*: vad fajok egyidejű kiaknázása a búzanemesítésben. *In: Veisz, O. (ed.): Növénynemesítés a megújuló mezőgazdaságban: XX. Növénynemesítési Tudományos Nap – 2014. március 18.* MTA Agrártudományok Osztályának Növénynemesítési Tudományos Bizottsága, Budapest, 314-318. p. (in Hungarian)

Mikó P., Megyeri M., Molnár-Láng M. (2013): Development of *Triticum timopheevii* derived synthetic amphiploid lines for wheat improvement. *In: Kőszegi, I. and Pósa, T. (eds.): Our Future – book of abstracts. – Pannonian Plant Biotechnology Conference for PhD Students in Plant Biology in Connection to the EPSO Fastination of Plants Day, 15. May 2013.* Pannonian Plant Biotechnology Association, Keszthely, Hungary, 25-26. p.

Mikó P., Megyeri M., Kovács G. (2011): Characterization of *Triticum timopheevii* gene bank accessions to gain useful materials for organic wheat breeding. *In: Veisz, O. (ed.): Climate change: Challenges and opportunities in agriculture. – AGRISAFE final conference, 21-23. March 2011.* Agricultural Research Institute of the Hungarian Academy of Sciences, Budapest, 90-93. p.

Conference abstracts:

Mikó P., Megyeri M., Molnár-Láng M., Kovács G., Rakszegi M., Hiltbrunner J., Löschenberger F. (2013): Comparison of different breeding strategies of cereals through different environments. In: Joint Meeting of EUCARPIA Section Organic and Low-Input Agriculture and EU NUE-CROPS Project, 24-26 September 2013. Georg August Universität, Göttingen, Germany, 23. p.

Mikó P., Megyeri M., Kovács G., Molnár-Láng M. (2013): Tetraploid wheat wild relatives as tools of wheat improvement. In: Ortiz, R. (ed.): Pre-breeding-fishing in the gene pool. Abstracts of oral presentations and posters of the European Plant Genetic Resources Conference 2013, 10-13 June 2013. NordGen and SLU, Alnarp, Sweden, 135. p.

Mikó P., Megyeri M., Kovács G. (2012): Development of *Triticum timococcum* to improve wheat breeding materials. EUCARPIA 19th General Congress. 21-24 May 2012, Budapest, 263. p.

Kovács G., Megyeri M., **Mikó P.**, Rakszegi M., Láng L. (2012): Organic breeding of einkorn (*Triticum monococum* ssp. *monococcum*): Development of semidwarf variety and its possible use in evolutionary plant breeding. EUCARPIA 19th General Congress. 21-24 May 2012, Budapest, 444. p.

Megyeri M., **Mikó P.**, Kovács G. (2012): A termesztett búza genetikai diverzitásának növelése alternatív gabona fajok felhasználásával. *In: Veisz, O. (ed.): XVIII. Növénynemesítési Tudományos Napok – Összefoglalók – 2012. március 6.* MTA Agrártudományok Osztályának Növénynemesítési Bizottsága, Magyar Növénynemesítők Egyesülete, MAE Genetikai Szakosztálya, Budapest, 108. p. (in Hungarian)

Mikó P., Megyeri M., Kovács G. (2011): Improvement of organic wheat breeding materials with the use of *Triticum timopheevii* gene bank accessions. ECO-PB Breeding Conference 2011: Organic Plant Breeding: What makes the difference? 3-4. November, 2011. Frankfurt am Main, Germany. *Abstract in proceedings: Lammerts van Bueren, E.T. et al. (eds.): Organic Plant Breeding: What makes the difference? 10 year's Anniversary Conference 2011.* Research Institute for Organic Agriculture (FiBL), Frankfurt, 44-45. p.

Mikó P., Megyeri M., Kovács G. (2011): Különböző *Triticum timopheevii* génbanki tételek vizsgálata organikus nemesítésre alkalmas alapanyagok előállítása céljából. *In: Matuz, J. et al. (eds.): XVII. Növénynemesítési Tudományos Napok – Összefoglalók – 2011. április 27.* MTA Agrártudományok Osztályának Növénynemesítési Bizottsága, Magyar Növénynemesítők Egyesülete, MAE Genetikai Szakosztálya, Budapest, 139. p. (in Hungarian)

Mikó P., Megyeri M., Kovács G. (2011): Increasing the genetic variability of *Triticum zhukovskyi* genetic resources via its reconstruction from the original genome donor species. EUCARPIA conference of the Genetic Resources Section, 5-7. April, 2011. Wageningen, The Netherlands. *Abstract in proceedings: van Hintum, T.J.L. (ed.): To serve and conserve, Abstracts of oral presentations and posters of the European Plant Genetic Resources Conference 2011,* CGN, Wageningen, 92. p.

Megyeri M., **Mikó P.**, Molnár-Láng M., Kovács G. (2011): Utilization of einkorn (*Triticum monococcum* L. ssp. *monococcum*) to widen the genetic diversity of cereals. EUCARPIA conference of the Genetic Resources Section, 5-7. April, 2011. Wageningen, The Netherlands. *Abstract in proceedings: van Hintum, T.J.L. (ed.): To serve and conserve, Abstracts of oral presentations and posters of the European Plant Genetic Resources Conference 2011,* CGN, Wageningen, 90. p.

Megyeri M., **Mikó P.**, Kovács G. (2010): Az alakor (*Triticum monococcum* ssp. *monococcum*) keresztezhetőségének vizsgálata tetraploid x diploid fajok interspecifikus hibridjeinek előállítása céljából. *In: Veisz, O. (ed.): XVI. Növénynemesítési Tudományos Napok – Összefoglalók – 2010. március 11.* MTA Agrártudományok Osztályának Növénynemesítési Bizottsága, Magyar Növénynemesítők Egyesülete, MAE Genetikai Szakosztálya, Budapest, 99. p. (in Hungarian)