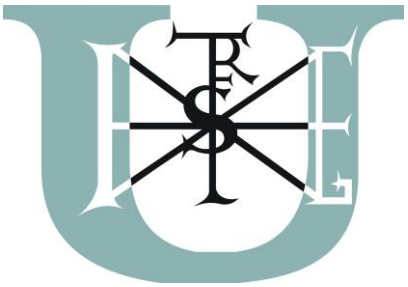

SZENT ISTVÁN UNIVERSITY

CYTOLOGICAL AND MOLECULAR BIOLOGICAL STUDY OF EGG CELL ACTIVATION IN CEREALS

Theses of PhD dissertation

DÁVID POLGÁRI

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Doctoral School: *Plant Science PhD School*

Director: Dr. Lajos Helyes, DSc
Professor, Department head
SZIU, Faculty of Agricultural and Environmental Sciences
Institute of Horticulture

Discipline: Crop Production and Horticultural Sciences

Supervisors: Dr. László Sági, *Ph.D.*
Senior Research Associate, Private Professor
Agricultural Institute, Centre for Agricultural Research,
Hungarian Academy of Sciences, Martonvásár

Dr. Katalin Jäger, *Ph.D.*
Department head
Agricultural Institute, Centre for Agricultural Research,
Hungarian Academy of Sciences, Martonvásár

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Dr. László Sági, *Ph.D.*
Supervisor

……
Dr. Katalin Jäger, *Ph.D.*
Supervisor

……
Dr. Lajos Helyes, DSc
Head of Doctoral School

1. RESEARCH BACKGROUNDS, AIMS OF THE RESEARCH

Due to the constant growth of the global population, the demand for food is in steady increase. Year after year, this poses an ever serious challenge to modern agriculture and the food industry. Consequently, plant breeding is becoming forced to test the limits of the genetic yield potential of the cultivated species. Furthermore, as a consequence of monoculture farming over huge areas, the intense use of pesticides, as well as climate change and global mobility, virulent pathogens and pests appear more frequently, to which cultivated varieties are not resistant. These pathogens and pests may cause enormous damage in a short time: in unfavourable years this may even lead to regional famines. For that very reason cooperation needs to be established between all related disciplines in order to prevent the gap between the supply and demand of basic foodstuff from further deepening.

For achieving constantly higher and more stable yields, the cultivated varieties need to be established on the widest possible genetic basis and possess high yield potential as well as excellent adaptability. As conventional breeding methods cannot ensure the rapid introduction of genes encoding new traits into the genome - or such solutions are not provided by conventional breeding at all -, significant progress can be achieved only by direct or indirect utilization of the genome or genetic information from other organisms. Fundamentally, this can be realised in two ways: by the direct insertion of isolated genes with gene technology or by sexual crossings between genetically distant species (interspecific crossings) and genera (intergeneric crossings).

The distant, wheat-alien hybrids are significant not only for their potential to renew the existing plant breeding pool, but they provide excellent models for revealing the molecular mechanisms that play a role in the cooperation between (distant) genomes and their inhibition. Imprinting and uniparental chromosome elimination belong to the latter mechanisms. Imprinting refers

to the permanent or temporary preservation in the developing offspring of the differential expression patterns coded by epigenetic regulation in the parental genomes. Uniparental chromosome elimination refers to the massive (partial or total) elimination of the chromosomes of one parental genome mainly during the mitotic cell divisions of early embryo development and the subsequent meiosis. Most of these hybrid plants are infertile, whereas those from the wheat × barley crossings are invariably sterile. The background of chromosome elimination is not fully understood but most likely it is provoked by the inharmonious parental cell cycles or the incompatibility between microtubules and histone proteins (Sanei et al. 2011). This phenomenon becomes even more interesting in that in some of the wheat-barley addition lines the paternal barley chromosomes become fixed in the next generation; whereas, in other lines, these chromosomes become destabilised again during the meiosis and only a few percent is passed to the offspring (Szakács and Molnár-Láng 2010). On one hand, the understanding of this phenomenon is of key importance for enhancing the efficiency of vertical gene transfer. On the other hand, it is important for expanding the boundaries of crossability, which is a tool for creating new, original combinations between species and genera.

It is clearly visible that beyond ensuring useful plant breeding stock, wheat-barley intergeneric hybrids have an even more significant role in pushing the present boundaries of cereals' crossability. In this sense they can turn into models to help understand intergenomic interaction and inhibition and, what is more, they can perhaps even lead to new discoveries. Therefore, in our research, our aim was to lay the systematic reproductive biological, biotechnological and genetic foundations of wheat × barley crossings and, based on that, to elaborate an efficient hybrid production system.

The experiments described in the thesis were planned along with the following specific objectives:

- Studying the main events of the progamic phase (pollen adhesion, germination and growth of pollen tube reaching the micropyle; release and transportation of male gametes, fertilisation of the female gametes) by light, fluorescent and confocal microscopy with special attention to the barley pollen - wheat pistil interaction.
- Studying the events in the embryo sac after the fertilisation (postgamic changes): comparing the processes in the self- and cross-pollinated ovules by light and confocal microscopy.
- Maximising the embryo and plant yield of wheat × barley crossings by selecting for the most crossable genotypes, combinations and supportive treatments.
- More efficient rescue and regeneration of hybrid embryos and the comparative histological examination of the hybrid embryos developing in planta.
- Analysing the genome composition of the wheat × barley F1 hybrids with special regard to the more profound understanding of chromosome elimination and inclusion.
- Selecting and characterising new dihaploid wheat lines that have improved crossability with barley.
- Answering the question whether it is possible to reduce or eliminate, by chemical treatments, the abortive defects characterising the embryo and endosperm development.

2. MATERIALS AND METHODS

2.1 Plant material

Wheat–barley crossings were carried out with three spring wheat (*Triticum aestivum* L.) cultivars as female parents: the international standard, 'Chinese Spring', another Chinese cultivar, 'Sichuan', and CS Ph^I, a high pairing derivative of 'Chinese Spring' containing the Ph^I gene of *Aegilops speltoides* (Tausch), which is a suppressor of the *Ph1* gene that controls homeologous chromosome pairing in wheat. The six-row spring malting barley (*Hordeum vulgare* L.) cultivar, 'Morex', was the primary male parent, whereas 'Golden Promise', a 2-row spring malting barley, was an additional male parent. As internal control for the confirmation of hybrids, a few crosses were also made with haploid-inducing *Hordeum bulbosum*. Control pollinations for crossability with rye were performed with three cultivars: 'Imperial', 'Mercator' (*Secale cereale* L.) and 'Kriszta', a perennial rye *(Secale cereale* × *Secale montanum*).

2.2 Intergeneric hybridisation

Two or three days before the controlled pollination, spikes of the maternal plants were emasculated and insulated with cellophane bags. Manual pollination was carried out on the day of anthesis. One day after pollination, 100 ppm of 2,4-dichlorophenoxyacetic acid was injected into the first internode below the spikes.

In order to reactivate the sex-specific, epigenetically inactivated genes, the plants were treated by demethylating agents suppressing the transcription of the methylation pattern. This was carried out at different times, usually in parallel with the 2,4-D treatment. Pseudo-seeds were removed from the pollinated spikes 14 days after the fertilisation and got sterilised. Developing embryos were rescued and placed on culture medium. Based on the number

and the maturity of the embryos, the conspicuous differences in the effects of the treatments were determined and the obtained data were statistically analysed (Polgári et al. 2014).

2.3 Micromorphological analysis after pollination

Living and dead pollens adhered to the stigma were made visible by fixing the pistils in Clarke's solution (1 part glacial acetic acid, 3 parts absolute ethanol), 0, 15, 30 and 60 min after pollination, and stained with trypan blue in 75°C water bath for 5 min.

For identifying the functional own and alien pollens, 10, 30 and 60 min after pollination, the pollinated pistils were fixed overnight in Clarke's solution. After washing in sodium phosphate buffer (pH 9), they were stained in 0.1% aniline blue solution for 1 hour, at room temperature. Stigmas were isolated from the stained pistils by chopping with a sharp lancet and studied by fluorescent microscopy.

For the in situ examination of the pollen tubes, fixed pistils were stained with aniline blue, dehydrated through ascending grades of alcohol and cleared in BABB solution (2 parts benzyl benzoate and 1 part benzyl alcohol). Cleared pistils were cut into halves and examined in a drop of BABB with confocal microscope.

For the in situ examination of embryo development, 24 and 72 hours after pollination, the pistils were dissected, split into halves and the released ovules were fixed in Clarke's solution. For labelling the nuclei, the samples were washed and stained by SYTO 63, a DNA-specific fluorochrome, dehydrated through ascending grades of alcohol and cleared in BABB solution.

The cleared pistils and pistil halves were viewed by confocal laser scanning microscope on a concave microscope tray, in a drop of 20 µL BABB using 1 mm silicone spacer. After HeNe laser-excitation, at the

required wavelength, of different fluorochromes, specific emission was detected with appropriate filters.

2.4 Molecular analysis of hybrid plants

Flow cytometry

Nuclei from young (5–7-day-old) leaves were isolated by chopping with a single-edged razor blade in 800 µL ice-cold isolation buffer. The crude nuclear suspension was subsequently filtered through 40 µm cell strainer caps. The fluorochrome propidium iodide (25 µg/mL) was left to intercalate into nuclear DNA for 3 min on ice. The measurements were performed by FACScan^{TM} flow cytometer.

Molecular marker analysis

In order to determine the genome composition of the progeny, specific primers to the male parental genome were designed and used for polymerase chain reaction on the isolated DNA template in a final volume of 20 µL. Ten µL of the PCR product was run on 2% agarose gel, followed by staining with ethidium bromide and recorded by gel documentation system.

Genomic (GISH) and fluorescent (FISH) in situ hybridisation

Anthers in the meiotic stage were fixed in Clarke's solution, prepared on microscope slide and stored at -20ºC. Barley total DNA was labelled with digoxigenin-11-dUTP by nick translation and used as a probe. GISH analysis was performed according to the description of Szakács et al. (2013). Unlabelled wheat DNA was used for blocking and added to the reaction in thirty-fold concentration of the probe. For the FISH analysis, GISH signals were removed from the slides in 50% formamide at 42°C, and re-hybridised with a $(GAA)_7$ probe specific to near-centromeric heterochromatin.

3. RESULTS

3.1 Cytology of progamic (prefertilisation) phase

No significant difference was found in the viability of wheat and barley pollen adhered to wheat stigma. At the first examination point – 15 min after pollination –, 44.6% of the wheat and 40.2% of the barley pollen was found viable. In the subsequent time points – 30 and 45 min after pollination –, the barley pollen again showed similar vitality to that of wheat: in the case of barley, 22.8% and 32.4%, in the case of wheat 24.3% and 35.3% of the pollen was found viable, respectively. Significant difference between the viability of wheat and barley was found only 60 min after pollination when the rate of the living wheat pollens (19.1%) was more than twice as much as that of the viable barley pollen (7.6%).

Wheat and barley pollen tubes alike managed to reach the micropyle in 1 hour. They even passed through the micropyle thus reaching the filiform apparatus of the synergid cells. Both types of pollinations also produced pollen tubes that did not enter the micropyle by the moment of fixation but they approached it to a few micrometers. Other pollen tubes were just heading to the micropyle in the space between the inner epidermis of the ovary wall and the dorsal side of the pistil.

3.2 Cytomorphology of the postgamic (postfertilisation) phase

Twenty dissected ovules were examined: in three ovules the first cell cycle of the zygote concluded normally as in the case of the control pistils. On one occasion, when the zygote was in the metaphase of the first mitosis, the mitotic spindle, the microtubules and the intensely condensed chromosomes lining up in the midline were clearly visible. In this case, the paternal chromosome elimination during early cell divisions “could be caught in the act” thus confirming the results stating that the barley chromosomes

sometimes lag behind due to the defective connections between centromere and microtubule. On one occasion, the phase immediately before fertilisation could be observed.

Most of the 3-day old embryos were in globular stage which means about a 1-day delay behind the drop-shaped control embryos. In every case, the endosperm showed only a few nuclei and undeveloped structure.

3.3 Production of intergeneric wheat-barley hybrids by 2.4-D treatment

'Sichuan' wheat genotype demonstrated the highest crossability with barley: plants could be grown from 14% of the pollinated flowers. 'Sichuan' was followed by 'Chinese Spring' Ph^I with 5.8% plant yield, while 'Chinese Spring' demonstrated the lowest results: viable plants were grown only from 2.3% of the pollinated flowers.

The plant population was analysed by diverse methods and the results were compared with each other for an overall evaluation. Altogether, 42 candidate hybrid plants were tested together with 6 *H. bulbosum*-derived haploid wheat plants as controls. In 85% of the cases (41 samples), application of at least two different methods brought evaluable results. In the hybrid population, the overall evaluation identified only 3 complete hybrids (7.1%, one by each three wheat genotypes). This means that, besides the 21 maternal chromosomes, they contained at least one copy of the 7 paternal chromosomes. Part of the paternal genome (at least 2 chromosomes) was found in 7 other plants (14.2%, also one by each three wheat genotypes).

Indications for parental genome rearrangements were revealed in 5 plants (11%, in four cases with chromosome marker and in one case with GISH). Genome composition could be determined precisely in 96% (46 plants) of the samples. In the case of all 10 plants tested by all 3 methods, 100% identical results were obtained.

3.4 Production of intergeneric wheat-barley hybrids by demethylation treatment

Three days after pollination no significant difference was found in the size and maturity of the self-pollinated control embryos and those in the demethylation-treated pistils. In both of the cases, the embryos consisted of 10-20 cells in average and took the shape of 50-60 μm long droplets. The most remarkable difference was shown in the size and appearance of the endosperm. In the treated pseudo-seeds fertilised by barley, the endosperm was very rarely present beside the embryos, and when present, it was, in most of the cases, an obsolete structure consisting only of a few nuclei and cytoplasm. In the case of two pseudo-seeds, the endosperm did not lag markedly behind the self-pollinated control in terms of size and maturity. Moreover, in one case, the endosperm was larger than the control and decidedly different in terms of its structure.

In the 6-day old treated pseudo-seeds fertilised by barely, the embryos were similar to the control embryos in their size and appearance; however, the endosperm was extremely varied from being totally absent to being completely cellularized.

Two weeks after pollination, most (81%) of the 2,351 pseudo-seeds resulted from barley pollination were already empty and contained neither embryos, nor endosperm.

In 78.3% (260) of the pseudo-seeds containing embryos (332), the embryos were markedly lagging in size behind those in the self-pollinated pseudo-seeds. In these pseudo-seeds the embryo-associated endosperm was never present. At the same time, however, in 66 cases (19.9%), the embryos were identical in size to those in the self-pollinated, control pseudo-seeds. Beside these embryos, the obsolete endosperm in gelatinous structure could always be found and as large as the embryo. Although only in 4 cases (1.2%), the endosperm filled the whole pseudo-seed and were similar to the control

but slightly smaller. These kinds of pseudo-seeds dried and crinkled after maturity, but upon interrupting dormancy, they germinated independently on damp filter papers and developed into viable plants. In two cases (0.6%), instead of the endosperm, other embryo-like forms (embryoids) developed in the pseudo-seeds.

Based on the chromosome(arm)-specific marker analysis of the 210 plants regenerated from the embryos, 80% of them contained at least 1 barley chromosome. No preference was revealed in terms of barley chromosome inclusion (and elimination); thus the process of chromosome elimination in the studied population can be regarded as random.

4. NEW RESULTS

- By comparing the viability of own (wheat) and barley pollens and the growth of the pollen tubes on the wheat stigma in the progamic phase of reproduction, no substantial difference could be established in the examined genotype combinations. Both of the wheat and barley pollens germinated similarly, grew pollen tubes and reached to the synergid cells through the micropyle on a number of occasions.
- In the progamic phase of reproduction, the development of the wheat × barley hybrid embryos gradually slowed down in comparison with the self-pollinated ones: 3 days after pollination, about a 1-day delay could be observed. As for the endosperm, the difference was even more remarkable.
- Further comparative studies on the self-pollinated and wheat × barley pseudo-seeds showed that the development of the latter seeds from 3 days after pollination onwards was delayed increasingly. There is substantial difference between the two studied groups: the development of the hybrid endosperm came on a standstill after some fast divisions which, after all, led to the total halt of the growth of the embryos. Between the 3rd and 14th day

after pollination, the hybrid embryos can be successfully rescued and grown up.

- By the application of new genotype combinations and chemical treatments, the highest ever embryo yield could be achieved, 30% in average (max. 41%), and plants could be regenerated from nearly four-fifths (78%) of the embryos. This efficient system of hybrid production is already adequate to carry out genomic studies in the future.
- In a large population containing 210 grown up hybrid plants, it was established by using molecular methods (flow cytometer, chromosome-specific DNA markers and GISH) that about 20% of the plants inherited both parental genomes, whereas at least one chromosome of the barley genome was eliminated in 60% of them. Finally, again in about 20%, maternal haploids were obtained as the paternal genome was totally eliminated probably still at the early stages of embryonic development. The above rates are far more favourable than it was described in earlier studies and they allow efficient hybrid production.
- By combining up-to-date microscopy analysis and cytogenetic insight, the phenomenon of chromosome elimination could be confirmed already in the 2-celled zygote and one barley chromosome (5H) lagging behind was successfully identified.
- By duplicating the genomes of haploid plants containing only maternal genomes, fertile dihaploid (DH) plants were produced. Originally, these plants were clearly generated by barley fertilisation and through uniparental genome elimination. Thus, they became homozygous for all those alleles that influence barley crossability. From these DH lines, new breeding material with better properties was selected.
- It was established that demethylation treatment delayed the breakdown of endosperm development and, in some cases, it could even be postponed to an extent that functional endosperm with normal anatomy developed. With

this method, for the first time, wheat × barley crossing resulted in autonomous caryopsis with germination ability.

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