Hungarian University of Agriculture and Life Sciences

MATE

MOLECULAR CYTOLOGICAL INVESTIGATION OF SELECTIVE CHROMOSOME ELIMINATION IN WHEAT × BARLEY HYBRID LINES

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INTRODUCTION - THE IMPORTANCE OF THE SUBJECT

Cereal production represents a leading sector of agriculture in both Hungary and. Europe, and worldwide. The major challenge of recent years in crop production is the unpredictable fluctuation of average cereal yield due to the higher frequency of extreme weather events. In a dry or extremely warm year, the crop failures may have a negative impact on the rate and predictability of the economic growth of a country.

In the era of climate change, genetic diversity within a species is of key importance to its adaptation to the complex co-occurrence of the new biotic and abiotic stresses. Wheat (*Triticum aestivum* L.) is a major crop used as human food and animal feed and is one of the earliest domesticated crops. During the long period of domestication, human selection for high-yielding traits eroded the genetic diversity of wheat, once present (and since maintained in the progenitor species) (Haas *et al.*, 2019; Venske *et al.*, 2019) The introduction of new genetic material into the wheat background, may potentially add agronomically desirable traits, which would broaden wheat's genetic diversity and resolve the "genetic bottleneck" created by domestication.

Interspecific hybridisation by spontaneous crossings or by directional cross-pollinations represents the traditional way of transmitting genes carrying useful traits into wheat. In wheat pre-breeding, sexually compatible relatives within the *Triticeae* tribe could be used. The diploid winter barley *(Hordeum vulgare* L.) can be harvested 7-10 days earlier compared to wheat, which would allow a faster grain development, avoiding the early summer drought periods. Wheat

breeding could also benefit from favourable nutritional traits of barley, such as essential amino acid (lysine) composition, fibre (β-glucan) and prebiotic content.

While attempts to generate a viable hybrid between wheat and barley (Hordeum vulgare L.) have a history of more than a hundred years, it remains a technically challenging process with a very low success rate. In addition, the transmission of the barley genome in the very few surviving embryos is further hindered by a process termed as uniparental chromosome elimination, that leads to the partial or complete loss of the chromosomes arising from the barley parent. Uniparental chromosome elimination may occur during the early embryonic cell divisions or during meiosis (Houben *et al.*, 2011; Ishii *et al.*, 2016), resulting in a haploid progeny. Despite its importance in chromosome transmission in wide hybrids the precise mechanisms behind chromosome elimination is yet to be revealed.

Centromeres are specialized chromosomal regions essential for chromosome movement and transmission during cell division. Accurate centromere function relies on the loading of the centromere-specific histone H3 (called CENH3 in plants) proteins into the centromeric DNA, ensuring an epigenetic control over centromere activity. The present study aimed to investigate the potential role of the CENH3 proteins in the uniparental chromosome elimination in wheat × barley hybrids. By discovering and eliminating the triggering factors of chromosome elimination, a new genetic source could be included into the wheat pre-breeding and breeding programs.

MATERIALS AND METHODS

1. HYBRID PLANT PRODUCTION

The wheat × barley F_1 hybrids analysed in the present study were obtained from a cross between the maternal doubled haploid line 'M1' (2*n*=6*x*=42), derived from the hexaploid spring wheat land race 'Sichuan', (Polgári *et al*., 2014) used as a female parent and the diploid two-row spring barley cultivar 'Golden Promise' (2*n*=2*x*=14) used as the male parent.

Crossings and subsequent embryo rescue at 14 days after pollination were performed, as described by Polgári *et al*. (2014). The parental plants and regenerated hybrid plantlets were grown in peat blocks (after a six-week vernalisation period at 4 °C and 12 h light) and then transplanted to pots in growth chambers (PGR-15, Conviron) or in growth cabinets (MLR-352-PE, PHCbi, Panasonic Corporation), respectively, under 16 h photoperiods.

2. MOLECULAR MARKER ANALYSIS

Because of the uniparental chromosome elimination, the obtained F_1 hybrid plantlets had been screened first, to determine their exact barley chromosome content, with a special regard to CENH3 coding chromosomes (1H and 6H), respectively. A PCR primer-pair set-up were designed, and PCR runs were applied for screening primary hybrids and identifying the present barley chromosome content of the F_1 plants.

3. ANTIBODY DESIGN, PRODUCTION, AND TESTING

Polyclonal antibodies recognizing the different CENH3 protein variants from wheat and barley were designed, produced, and tested for specificity by Immunolabelling. Then used as a tool to analyse cross-species incorporation of the CENH3 protein variants in primary wheat-barley hybrids.

4. GENERATING CENTROMERE SPECIFIC FISH PROBES

To study the interspecific loading of CENH3, in addition to visualizing the CENH3 protein, we needed to be able to distinguish the two centromeric regions of chromosomes originating from the different parental species. Despite the high degree (85%) of similarity between the centromere-specific sequences of the two species, we were able to isolate parental centromeric sequences that could be considered unique. We amplified centromeric FISH probes in PCR-reactions. Then different fluorescent pigments were conjugated to them by direct labelling, and their specificity was confirmed by *in situ* hybridisation.

5. OPTIMISATION OF A NEW MICROSCOPE SLIDE PREPARATION METHOD

An easy-to-use plant nuclei preparation method was developed with the aim of producing high-quality nuclei preparations that simultaneously preserve the 3D ultrastructure of the nuclei and ensure the access of antibodies to the epitope. Desired results were achieved by shortening the fixation time and by more efficient penetration of the fixative to the inner cell layers of intact tissues, by adding a

detergent (IGEPAL) to the non-denaturing fixative (4% PFA). Furthermore, by optimising the fixation time, the proportion of damaged cells and cell debris on the preparations was reduced, especially in wheat. Therefore, this method was considered as the optimal fixation time for the genotypes used in the present study. The cell wall and cytoplasm were mechanically removed using a dedicated tissue grinder tool set, which allowed to process large amounts of plant material in a short time, and to produce a large number of nuclei preparations, compared to the previously used enzymatic digestion and manual maceration methods. Using LB01 buffer for in-solution tissue homogenizing, resulted in optimal separation of nuclei and the least amount of cell aggregates. The optimized method has been published (Publication see in Results).

6. CYTOLOGIC AND IMMUNO-HISTOCHEMICAL ANALYSES

Considering the barley chromosome content, two of sixteen F1 hybrid plants were selected and examined by further ImmunoFISH analyses to determine the species specific-, and cross-species incorporation of CENH3 in mitosis and meiosis, and to follow the nature of chromosome elimination process in wheat × barley primary hybrids in mitosis and meiosis.

7. INVESTIGATION OF CENH3 LOADING IN THE MITOTIC CELLS OF THE F_1 HYBRIDS

To reveal whether wheat and barley centromeres (identified with FISH) in the hybrid nucleus have the capacity to mutually-incorporate in a cross-species manner CENH3 proteins (identified with immunolabelling), or they only load endogenous CENH3

proteins, the ImmunoFISH, method was used, combining simultaneous immunolabelling of CENH3 protein, together with centromere-specific CRW (wheat)-, and G+C (barley), that probes were selectively detected.

8. MICROSCOPY

Confocal laser scanning microscope was used for simultaneous detection of immunolabelled CENH3 protein variants and their incorporation into the parental centromeric DNA sequences.

RESULTS

1-2. PLANT MATERIAL AND MOLECULAR MARKER ANALYSES

14 days after crossings, and a subsequent embryo rescue and plant regeneration, the F1 hybrid plantlets were screened by molecular marker PCR analysis, that revealed their individual and exact content of the present paternal barley chromosomes. Based on the results, we selected two primary hybrid plants that could be used for the purpose of our research.

3. ANTIBODY DESIGN, PRODUCING AND TESTING

Three out of the designed four polyclonal antibodies (Wheat α, Wheat β, Barley α) proved to be suitable for the detection of species-

, and variant specific incorporations of the targeted CENH3 proteins in the root tip nuclei isolated from wheat × barley hybrid plants.

4. GENERATION OF CENTROMERE-SPECIFIC WHEAT AND BARLEY FISH PROBES

Visual separation of the parental centromeres was enabled in the hybrid nucleus by the centromere-specific FISH assays and used in loading studies of CENH3 protein variants in mitotic and meiotic cells of the F1 hybrid plants.

5. OPTIMISED NEW MICROSCOPE SLIDE PREPARATION METHOD

A new optimised microscope slide preparation method was elaborated, that efficiently preserves the 3D nucleic structure and enables the access of antibodies to the epitope. Moreover, this preparation method allows the combined usage of *in situ* hybridisation and immunolabelling methods, applied simultaneously (Makai, Diána - Mihók E.*et al.*, 2023).

6. CYTOLOGICAL CHARACTERISATION OF TWO WHEAT × BARLEY F_1 HYBRIDS

Based on the barley chromosome content of the sixteen F1 hybrid plants produced, two plants were selected for further analyses, both showing a unique chromosome composition, with a special regard of presence and absence of the two barley chromosomes, carrying the barley CENH3-encoding genes (1H and 6H).

Hybrid No. 22/2020 was characterised a **"partial hybrid"** with respect to the barley genome, as it was found to contain a partial set

of barley chromosomes, based on barley **chromosome-specific marker analysis**, that found 4 barley chromosomes (3H, 4H, 5H and 6H).

Hybrid No. 22/2020 was characterised as a **"partial hybrid"** with respect to the barley genome, as it was found to contain a partial set of barley chromosomes, based on barley **chromosome-specific marker analysis**, that found 4 barley chromosomes (3H, 4H, 5H and 6H), which only one (6H) was a Barley CENH3β-encoding, but Barley CENH3α-encoding 1H was not present).

(Barley CENH3α- and β+)

Cytogenetic analyses identified an additional 21 wheat chromosomes in addition to the four barley chromosomes, i.e., the complete maternal haploid genome was also present in the hybrid.

Cytological examination of root tip cells (n=75) using barley genomic DNA and 5S rDNA as probes by simultaneous GISH-FISH revealed 21 wheat and 4 barley (3H, 4H, 5H and 6H) chromosomes, and confirmed the absence and presence of two barley chromosomes encoding CENH3 variants: the absence of 1H encoding Bα, and the presence of 6H encoding CENH3β, in somatic cells of the hybrid.

Hybrid No. 28/2020 was characterized a **"complete hybrid"** with respect to the barley genome, as chromosome marker analysis and *in situ hybridization* of the hybrid genome identified all 14 barley chromosomes, revealing duplication of the paternal genome (n=48). In addition to the barley chromosomes, a variable number of wheat

chromosomes (0-20) were detected. In the majority (79%) of the cells analysed, the number of wheat chromosomes varied between 14 and 20, indicating mitotic instability and elimination of wheat chromosomes. In a subset of the mitotic nuclei analysed (21%), only chromosomes from barley parents were retained, while wheat chromosomes were completely eliminated.
(Barley CENH3α^+ and β^+)

7. CENH3 LOADING AND NUCLEAR ORGANISATION IN THE MITOTIC CELLS OF THE F1 HYBRID NO. 22/2020

F_1 hybrid **No. 22/2020**, carrying the full haploid chromosome component of wheat (21 chromosomes) and four chromosomes from barley, which included the 3H, 4H, 5H and 6H chromosomes. The absence of the 1H chromosome (presumably eliminated during the embryonic cell divisions) implied that the gene encoding the Bα CENH3 histone protein is lacking.

To reveal whether wheat and barley centromeres have the capacity to mutually- incorporate cross-species CENH3 proteins or they only load endogenous CENH3 proteins, we performed ImmunoFISH with CRW and G+C probes and species and variant specific CENH3 antibodies. We have selectively detected the wheat and barley centromeres but the Bα CENH3 antibody failed to produce immunosignal within the centromeres in the somatic nuclei analysed (Figure 17), which confirmed the absence of Bα CENH3 protein, in the wheat × barley F1 hybrid No. 22/2020.

In further experiments, immunoFISH with CRW and G+C probes and Wα or Wβ CENH3 immunofluorescence revealed a variable number 11-24 wheat centromeric signals and 2-4 barley centromeric signals (n=44). The number of wheat and barley centromeric signals indicated associations between the barley centromeres and similar associations between the wheat centromeres. In some cases, barley centromeres partially colocalised with wheat centromeres, revealing association between the centromeres of the two parental species.

The Wα and Wβ CENH3 signals colocalised with the CRW sequences of the wheat centromeres, revealing normal maternal centromere activity. Similarly, Wα and Wβ CENH3 histones colocalised with the G+C signals of the barley centromeric DNA, irrespective whether they were located individually. This indicated that the maternal CENH3 histone variants are loaded into both the wheat and the barley centromeres in the wheat × barley F1 hybrid No. 22/2020, revealing that barley centromeric repeats are capable to load CENH3 proteins from a related species.

8. CENH3 LOADING AND NUCLEAR ORGANISATION IN THE MITOTIC CELLS OF THE F1 'FULL' HYBRID NO. 28/2020

F1 hybrid **No. 28/2020**, carrying 14 barley chromosomes, representing the full diploid genome of barley (1H-7H) beside a variable number (0-20) of wheat chromosomes. (CENH3 Bα+, CENH3 Bβ+)

ImmunoFISH with Wα, Wβ or Bα CENH3 immunofluorescence and the hybridisation of CRW and G+C wheat and barley centromere-

specific probes revealed a variable number of wheat centromeric signals ranging from 0-19 along with 7-14 barley centromeric signals arranged into one group close to the nuclear periphery (n=45). Wheat centromeres localised at the periphery of the centromere group, surrounding the barley centromeres. The number of barley centromeric signals corresponded to that counted for the barley somatic cells, revealing that barley centromere-centromere associations take place in the hybrid nuclei as well. The large variation in the number of wheat centromeric signal and in some cases their complete lack pointed to the progressive elimination of the wheat chromosome set. This coincided with a less intense or missing Wα CENH3 signal within the wheat centromeres. In contrast, barley centromeres showed a clear Wα CENH3 signal, indicating that Wα CENH3 is transcribed in the hybrid nuclei. Wβ CENH3 signals colocalised with both wheat and barley centromeres and their number ranged from 8 to 22 (Figure 18), indicating that centromere-centromere associations occur in the somatic nuclei of the F1 hybrid No. 28/2020. ImmunoFISH with Bα CENH3 antibody revealed loading of Bα CENH3 histone protein into the barley centromeres, however no immunolocalisation could be detected within the wheat centromeres by our methodology.

9. CENH3 LOADING AND CENTROMERE ORGANISATION IN MEIOTIC CELLS OF WHEAT × BARLEY F1 HYBRIDS

To show if wheat CENH3 variants are cross loaded into the barley centromeric repeats in the meiotic cells as well, we performed immunoFISH on meiotic prophase I nuclei of the wheat × barley F1 hybrids No. 22/2020 and 28/2020. In the hybrid No. 22/2020, barley

centromeric signal marked by the G+C repeat probe ranged from 2-4, similarly to that observed in the mitotic cells. The number of wheat centromeric signal ranged from 11-20 (n=18, Figure 19). Our results confirmed that Wα and Wβ CENH3 protein signals colocalised with both wheat and barley centromeres within the meiocytes of the partial hybrid No. 22/2020.

In the meiotic cells of the hybrid No. 28/2020 only barley centromeres could be detected, revealing complete elimination of the wheat chromosomes (Figure 20). The number of barley centromeric signals ranged between 5-13. The signal of the wheat W α W β B α CENH3 CRW GC-repeat Merge + DAPI 57 specific CENH3 protein variants (Wα and Wβ) could not be detected on any of the meiotic samples analysed whilst Bα CENH3 colocalised with the barley centromeres.

DISCUSSION

The present study aimed to understand the incorporation of the parental CENH3 histone proteins in newly developed wheat × barley F1 hybrids and its role in uniparental chromosome elimination. The research project required optimization of molecular cytology techniques to be able to produce a large amount of high-quality plant cell nuclei, suitable to routinely perform complex molecular cytology examinations. Several critical factors had to be optimized before the procedure could be applied consistently.

The new plant nuclei preparation procedure made it possible to combine immunolabelling of CENH3 variants with 3D FISH that

allows the detection of multiple centromeric DNA sequences, thus differentiating between the wheat and barley centromeric DNAs even when enclosed in the cell nucleus. This approach opened the possibility to assess the incorporation of wheat and barley CENH3 protein variants into the wheat and barley centromeric DNAs in wheat × barley hybrids at the single cell resolution.

Two wheat × barley F1 hybrids were produced and tested for the cross-species incorporation of wheat and barley α– and βCENH3 protein variants into the centromeric DNAs. Wheat and barley α– and βCENH3 variant loading were evaluated in hybrid mitotic nuclei by a combination of centromere-specific repetitive DNA probes and species and variant-specific CENH3 antibodies.

Our study confirmed the absence of barley αCENH3 protein from hybrid 22/2020 and showed that wheat CENH3 variants could be incorporated into both wheat and barley centromeres. The retention of the four barley chromosomes and their maintenance through consecutive mitoses in the hybrid No. 22/2020 indicated that barley chromosomes can be stably inherited despite the lack of the con-specific CENH3 protein variants.

The cereba retroelement is highly conserved within the grass centromeres (Presting et al., 1998; Qi et al., 2013). Individual copies of the barley *cereba* and its wheat orthologue CRW share a sequence homology of 85% (Liu et al., 2008). The high level of homology between the barley *cereba* and the wheat CRW retrotransposons may contribute to the succesful incorporation of wheat CENH3 protein into the barley centromeres. Although barley centromeres are also interspersed with inherently different repetitive sequences, such as the G+C-rich satellite, the interaction between the centromeric DNA and

CENH3 proteins within the *Triticeae* tribe is not entirely conservative as CENH3 can be deposited into neocentromeric repeats completely absent from the native centromere (Nasuda et al., 2005).

SUGGESTIONS

Further analysis of epigenetic and genetic features of centromeres within a wider range of cereal species is needed to understand and influence chromosome stability/elimination in crop improvement programs. Understanding the driving force of the phenomenal evolution rate of centromeric DNA and CENH3 proteins would be applicable to plant breeding, allowing the production of hybrid combinations so far unreachable for crop improvement.

NEW SCIENTIFIC RESULTS

1. The present work developed a new plant nuclei preparation method suitable for the high-quality single-cell analysis when combining it with a range of most up-to-date molecular cytology and microscopy methods (e.g., immunochemistry, *in situ* hybridisation, immunoFISH, immunoGISH).

2. The presented research project effectively developed and tested for the first time a palette of specific polyclonal antibodies suitable to detect three of the wheat and barley CENH3 protein variants. The obtained protein antibodies are suitable for species-specific detection

of CENH3 protein variants in wheat × barley hybrid lines and their derivatives.

3. Combining molecular cytology with high resolution microscopy, barley and wheat centromeric DNA was visualized simultaneously with the respective CENH3 protein variants in the three-dimensional structure of the mitotic and meiotic cell nuclei of wheat-barley F_1 hybrids. This allowed to study endogenous and cross species incorporation of the different CENH3 histone protein variants.

4. The present work revealed for the first time that barley centromeric DNA has the capacity to load both CENH3 protein variants of wheat and that of barley CENH3 a during the mitotic cell cycle of the wheat × barley F1 hybrids. This work showed that barley chromosomes follow a stable chromosome inheritance even when one of the endogenous, barley CENH3 variants is unavailable within the hybrid cell nucleus.

5. The plasticity of barley centromeric DNA to load wheat CENH3 proteins has been detected in meiotic cells, and barley chromosome stability was confirmed during meiotic prophase I of the F1 hybrid.

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