HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES (MATE)

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

PhD Dissertation

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Doctoral School: *Plant Sciences*

Head of School: *Prof. Lajos HELYES*

Supervisors: *Adél SEPSI PhD, László SÁGI PhD*

Centre for Agricultural Research,

Martonvásár, Hungary

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Approved by Head of School Approved by Supervisors

Abbreviations

Bα: anti-barley αCENH3 variant antibody

Bβ: anti-barley βCENH3 variant antibody

BC1: first backcross generation

CENH3: centromeric histone type 3 (H3) protein

CENP-A: centromere protein A

CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

CTAB: hexadecyltrimethylammonium bromide

DAPI: 4',6-diamidino-2-phenylindole

DNA: deoxyribonucleic acid

FISH: fluorescence *in situ* hybridisation

GISH: genomic *in situ* hybridisation

G1 phase: the gap 1 or growth 1 phase of the cell cycle

G2 phase: the gap 2 or growth 2 phase of the cell cycle

HJURP: Holliday junction recognition protein

ICC: immunocytochemistry

M phase: the mitotic phase of the cell cycle

MTOC: microtubule-organising centre

PBS: phosphate-buffered saline

PFA: paraformaldehyde

TNBg: TRIS-Na Blocking buffer with glycine

TRIS: tris(hydroxymethyl)aminomethane

S phase: the synthesis phase of the cell cycle

SSC: sodium-saline citrate

Wα: anti-wheat αCENH3 variant antibody

Wβ: anti-wheat βCENH3 variant antibody

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2 INTRODUCTION

Cereal production represents a leading sector of agriculture in Hungary, 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-occurence 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 the wheat 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 have a history of more than hundred years, it remains a technically challenging process with a low success rate. In addition, the transfer of the barley genome via the few surviving embryos is further hindered by a process termed uniparental chromosome elimination, which leads to the partial or complete loss of the chromosomes 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 mecanisms behind chromosome elimination is yet to be revealed.

Centromeres are specialised chromosomal regions essential for chromosome movement and transmission during cell division. Accurate centromere function relies on the loading of the centromere-specific histone H3 (CENH3) proteins into the centromeric DNA, ensuring epigenetic control over centromere activity. The present study aimed to investigate the potential role of the CENH3 proteins in the process of 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 programmes.

3 LITERATURE REVIEW

3.1 Genome organisation in the cell nucleus

The universal carrier of the genetic information within prokaryotes and eukaryotes is the DNA molecule. Every cell of a eukaryotic organism carries its DNA inside the cell nucleus (in the form of double-stranded fibres) and in the cell organelles, as mitochondria and plastids (a circular formed DNA), together is called chromatin. The eukaryotic cell cyle includes two distinct phases: the interphase that includes much of the metabolic activities, characteristic for the cell's differentiation and the subsequent mitotic phase, which includes the cell division. In the course of the cell cycle, the chromatin undergoes dynamic structural changes, condensations and decondensations, continually serving the stage-specific gene expressions. Most of the chromatin in the interphase (which includes the G1, S, and G2 phases) is decondensed (termed euchromatin), staying accessible for the gene expression machinery. However, certain regions of the chromatin remain in a condensed form (termed heterochromatin) lacking gene expression activity. In the mitotic phase, the previously duplicated chromatin organises into its most condensed form, the metaphase chromosome, which segregates during cytokinesis into two daughter cells.

The basic structural subunit of the chromatin is a solenoid nucleosome complex. The nucleosome complex contains a 146-bp condensed chromatin fiber rounded up on a histone octamer protein complex: each of the canonical H2A, H2B, H3, H4 histone proteins in two copies makes the spindle for the DNA to coil up (Nakayama *et al.*, 2001; Grewal and Moazed, 2003; Black *et al*., 2004; Lermontova *et al*., 2015; Pentakota *et al*., 2017). The specific amino acid sequences (so-called „histone-fold domains") of the histone proteins ensure the fixation of the bonds to the coiled DNA chains. The extrinsic N-terminal regions of histones are the locations of the epigenetic modifications (methylations, acethylations, phosphorylations, sumoylations and others) (Allshire and Karpen, 2008). As a consequence, histone proteins determine the entire euchromatin-heterochromatin organisation and thus influence the actual gene expression pattern in the cell (Grewal and Moazed, 2003; Lermontova *et al.*, 2006).

3.2 Chromosome structure and inheritance

3.2.1 Landmark chromosome regions

The entire metaphase chromosome set visually represents (size, shape, arms etc.) the genome of the corresponding species.

The landmark regions of the chromosomes are:

1) The primary constriction of the chromosome is the centromere that enables the attachment of the spindle microtubules, essential for chromosome segregation (see Figure 1A). More than a hundred years ago, in 1882 Walter Flemming observed under a light microscope and published the centrally localised primary constriction of the mitotic chromosomes (Flemming, 1882).

2) Chromosome arms are the condensed chromatin fibers located one- or bidirectionally from the centromere, starting from the flanking region(s). Depending of the ratio of the length of the chromosome arms several types of chromosome arm compositions can be determined: metacentric, submetacentric, acrocentric and telocentric.

3) The telomere is a chromosomal region bearing repetitive nucleotide sequences associated with specialised proteins at the ends of linear chromosomes.

4) Satellite DNA is part of the distal region of a chromosome that is differentiated from the rest of the chromosome by a secondary constriction.

Special cytogenetical methods have been developed to identify the particular chromosomes of various species by different staining methods (Giemsa staining, C-banding, Q-banding, FISH method).

3.2.2 The centromere in general

The centromere is a special chromosomal region that can be identified by its unique and highly conserved function: enabling the chromosomes to attach the microtubules and ensure the proper chromosome segregation during the cell cycles. It represents a necessary and essential role in correct inheritance of the genetic material through generations. Independently of the diversity found in size, organisation, and structure of centromeric DNA, many examples - a point centromere in *Saccharomyces cerevisiae* a 125 bp short regional in *Candida albicans* 3-5 kb (Sanyal *et al.*, 2004; Earnshaw *et al.*, 2013), long regional in *Drosophila melanogaster* 420 kb, and in *Homo sapiens* - holocentric in metazoa, *Caenorhabditis elegans* 14-21 Mb (Monen *et al.*,

2005) i.e., diffused over the entire chromosome, - prove its necessity for proper microtubule binding (Balzano and Giunta, 2020). The centromere is embedded into the heterochromatic pericentromere (Lermontova *et al.*, 2015). The chromosomes are organised into adjacent sister-chromatids, that are fixed longitudinally by highly densed tetrameric cohesin proteins (Allshire and Karpen, 2008; Dalal *et al.*, 2008).

The plant centromeric DNA is a noncoding region, having similar strucural properties within different species. It consists mostly of long terminal repeats (LTR) retrotransposons and other shorter specific repetitive DNA motifs as „satellite", or tandem repeats. Centromeric repeat sequences are rich in A/T nucleotides and are arranged in tandem occurrence in many species (Choulet *et al.,* 2010; Ravi *et al.,* 2010; Ravi *et al.,* 2011). Although the primary structure (nucleotide sequence) of the centromeric DNA may resemble in motifs between distant species, the exact base sequence is not a determining factor, since different centromeric DNAs from different species can fulfill the same centromere function. Even within a single species a DNA sequence lacking the characteristic centromeric satellite DNA can function as a centromere. Human neocentromeres (Koch, 2000) and neocentromeres in plants (Guo *et al.*, 2016) serve as important examples.

The CENH3 protein

The centromere are the clusters of specific nucleosomes, that instead of canonical H3 histone protein, contain the centromere-specific variant of the H3 histone protein (CENH3 in plants, CENP-A in human, Cse4 in fungi, Cid in flies) (Batzenschlager *et al.,* 2015). The CENH3 associated nucleosomes are interspersed within those containing the canonical H3 protein variant in the centromeric region. The crystal structure of the CENH3 nucleosome is similar to the conventional nucleosome structure. The primary coil of the centromere specific nucleosome contains a histone octamer consisting of four histone protein units: H2A, H2B, CENH3, H4 in two copies (Luger *et al.*, 1997).

Specific amino acid sequences of CENH3 ensure the fixation of protein dimers inside the nucleosome core. Further protein-protein bonds and protein-DNA interactions compact and fix the DNA configuration (Luger *et al.,* 1997; Allshire and Karpen, 2008; Black and Bassett, 2008; Bailey *et al.*, 2013; Goutte-Gattat *et al.*, 2013; Fachinetti *et al.*, 2015; Muiruri *et al.*, 2017).

The amino acid sequences of the CENH3 proteins show homeology in the characteristic structural domains of the histone protein family along the diversity of species. The most conserved region is at the carboxyl terminal domain. One of the conserved subunits serves as a centromere targeting domain (CENP-A targeting domain, CATD, including the α2 helix and the L1 loop), which conducts the centromeric localisation of CENH3. The most variable amino acid sequences were found at the N-terminal region. The amino-terminal „tail" is an unstructured, linear amino acid chain, floating outside of nucleosome, and serves as the locus of the epigenetic regulations (methylation, acethylation, phosphorylation) (Black *et al.*, 2004; Goutte-Gattat *et al.*, 2013; Maheshwari *et al.*, 2015; Britt and Kuppu, 2016; Allu *et al.*, 2019).

The CENH3 cycle

The loading of the canonical histones occurs periodically during the genome replication in S phase. In contrats to the canonical histones, CENP-A is not expressed and reloaded during the S phase of the cell cycle (Shelby *et al.*, 2000). In the S phase the preexisting CENP-A nucleosomes are equally distributed on the two newly synthetised DNA strands and the gaps are then filled with the canonical histone nucleosomes. A CENP-A expression takes only place in the subsequent G2 phase (Müller and Almouzni, 2014).

Recent findings revealed the crucial role of a chaperone protein molecule called HJURP (Holliday junction recognition protein) in the process of CENH3 assembly into the centromeric DNA. This molecular machinery differs from those identified for canonical H3 histone loading (Foltz *et al.*, 2009; Dunleavy *et al.*, 2009). The newly expressed CENH3s are helped by HJURP to direct their centromere targeting domain to the DNA. During later phases the deposition of CENH3 initiates in several steps. The deposition of the newly synthetised CENP-A occurs in the late M (in telophase, when the chromatids decondense) and G1 phases. Firstly, the Mis18α, Mis18β chaperones transiently associate with the centromeric chromatin, as nucleosome clusters form rosette-like structures around HJURP during G1 (Andronov *et al.*, 2019), without being associated with CENP-A. This suggests that the chromatin „prepared" in a such way is ready for „receiving" the CENP-A, presumably owing to phosphorylated N-terminal tails, and trimethylated H3 lysine 9 (H3K4me2) or transcription of *centromeric alphoid* DNA (Allshire and Karpen, 2008; Dunleavy *et al.*, 2009; Foltz *et al.*, 2009). The second step is the exact depostition of CENP-A on

the centromeric chromatin. This step is mediated by HJURP at the presence of histone H3K4me2, that triggers the maturation of the pre-nucleosomal complex, and turns a CENP-A:H4 tetramer into a full octameric nucleosome in the first part of G1 (Allu *et al.*, 2019). The last step is the stabilisation of the freshly incorporated nucleosomes by protein-protein and a DNA-protein junctions (Lagana *et al.*, 2010).

The *active centromere* or the functional centromere is the initiation site for kinetochore assembly (Hirose *et al.*, 2011). It contains the regularly recruited CENH3 nucleosomes beside the canonical H3 (Earnshaw and Rothfield, 1985). It represents a direct binding site for the kinetochore proteins and is sufficient for the recruitment of the kinetochore-complex (Lermontova *et al.*, 2006).

CENP-A indirectly affects the spindle checkpoint functions (Black and Bassett, 2008). Any abnormality in CENH3 expression or recruitment can lead to mitotic segregation defects, as aneuploidy caused by chromosome elimination.

Among the members of the *Triticaeae* tribe two variants of the CENH3 protein exists: the αCENH3 and βCENH3. In the genome of hexaploid bread wheat there are three, slightly different copies of each CENH3 protein variant encoding genes, derived from the A, B, and D genomes. All six genes localise on the homoeologous group 1 chromosomes (Li *et al.*, 2013; Yuan *et al.*, 2015). The diploid barley genome encodes αCENH3 on chromosome 1H, and βCENH3 on chromosome 6H (Sanei *et al.*, 2011; Ishii *et al.*, 2016).

The Kinetochore-complex

The kinetochore-complex is a lamelle-formed protein complex recruited onto the active centromeres of the condensed metaphase chromosomes (Figure 2). In most eukaryotes, the kinetchore-complex is a docking site for the microtubules. It ensures the dynamic spatial segregation of sister chromatids (Figure 3B) into the daughter cells (Cheeseman and Desai, 2008; Dalal, 2009; McKinley and Cheeseman, 2016).

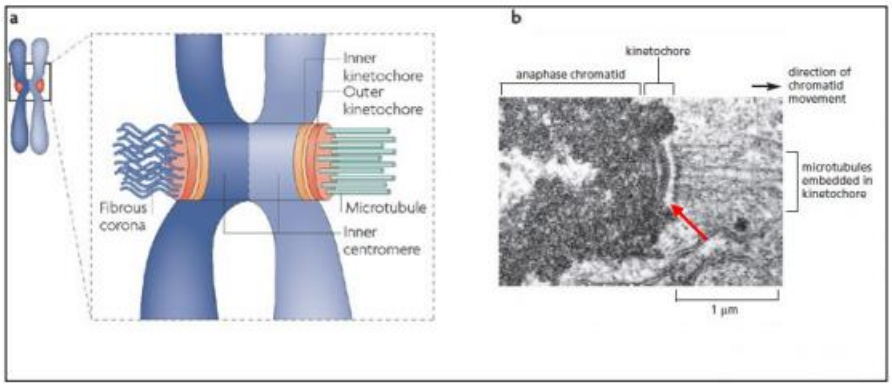


Figure 1. Schematic structure of the central region of the chromosome.

(A) *It includes the centromere and the two-layered kinetochore complex connecting microtubules.* ***(B)*** *The inner and outer kinetochore plate (red arrow) and the microtubules in the electron microscopic image can be well observed. (Source: Cheeseman* et al.*, 2008)*

The canonical kinetochore-protein molecules are deposited and complexed into a large macromolecule structure on the outer side of the centromeres of the cohesed sister chromatids (Cheeseman and Desai, 2008). Beyond the nucleosomes, the CENH3 is a common building block of the inner plate of the kinetochore-complex as well (Allshire and Karpen, 2008). An 80-100 different protein components were identified and their organisation into a higher order kinetochore structure has been studied (Cheeseman and Desai, 2008). Most of the constitutive kinetochore proteins, and the putative assembly factor were identified and characterised in *Arabidopsis thaliana* and their homologues were reported in barley (Lermontova *et al.*, 2015).

The underlying base for the recruitment of kinetochore macromolecule structure, is the active centromere that contains CENH3 (CENP-A) nucleosome clusters interspersed within the canonical H3 containing nucleosome clusters. These nucleosomes are organised into a globular higher order structure, that is shaped by epigenetical modifications (Black and Bassett, 2008). The methylated H3 histone (H3K4me2) containing centromeric nucleosomes are arranged and localised into a discrete internal domains (the inner sides) of the condensed centromeric regions of metaphase chromatids. The globularly-folded CENH3 containing nucleosomes (called CENP-B boxes) are sorted on the outermost regions (on the surface) of the condensed centromeres (Houben *et al.*, 2007; Lermontova *et al.*, 2015). CENP-A containing nucleosomes thus form the site for the recruitment of the CENP-A containing kinetochore-proteins (Amor *et al.*, 2004; Cheeseman and Desai, 2008; Earnshaw *et al.*, 2013) .

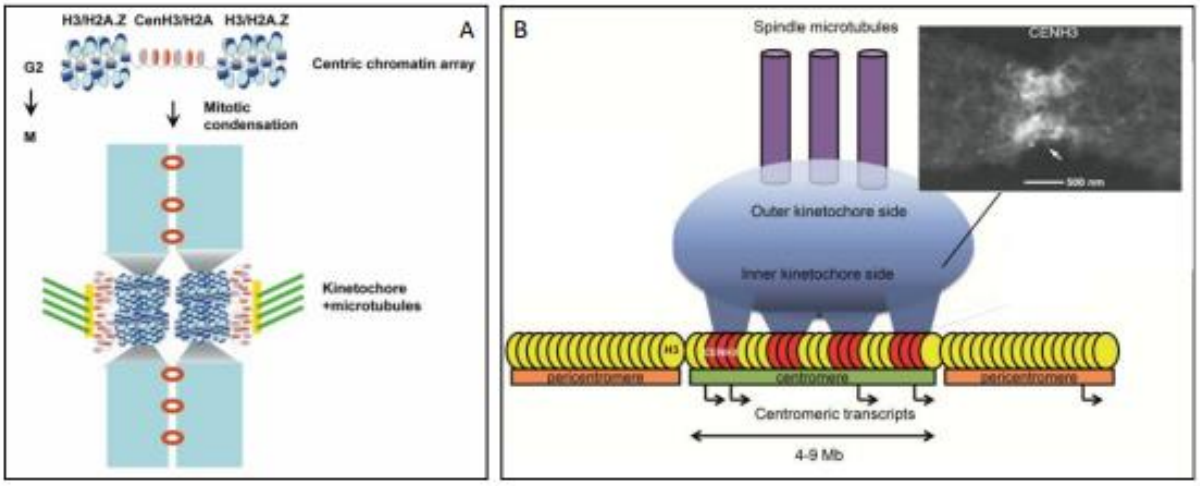


Figure 2. Simplified models of centromere.

(A) *The two chromatids (light blue) are laced together by tetrameric cohesin proteins (red), while H3 (blue-gray) and CENH3 (pink-gray) are connected to the centromere region containing nucleosomes intervolved with histone proteins, with microtubules (green) stitched yellow lamelled-like kinetochore complex. (Source: Dalal, 2009)*

The initial recruitment of the CENH3 containing kinetochore proteins is followed by the incorporation of other CENH3-containing kinetochore proteins, shaping up the inner kinetochore plate domain, which interacts with the cromatin. The kinetochore recruitment is terminated by emerging of the outer kinetochore plate, that serves as a transfixion site to the microtubule attachment (Allshire and Karpen, 2008). That specific „dual localisation" of CENH3 accomplish the connection between the centromere and the kinetochore, emphasising the crucial role of CENH3 during the correct chromatid segregation processes. Any failure of the correct kinetochore assembly, or microtubule attachment, or the sister cromatid-cohesion disorder can lead to segregation errors, causing a vital error in the transmission of the genetic material to the progeny.

Mechanisms of chromosome movement

In contrary to the animal kingdom, plants do not posess an organised centriole and a centrosome, nor animal dyneins to conduct the accurate distribution of the replicated genetic material. A mitotic spindle-shape stucture forms out from microtubules, generating a 25 nm diameter frame of polymers of α- and β-tubulin dimers.

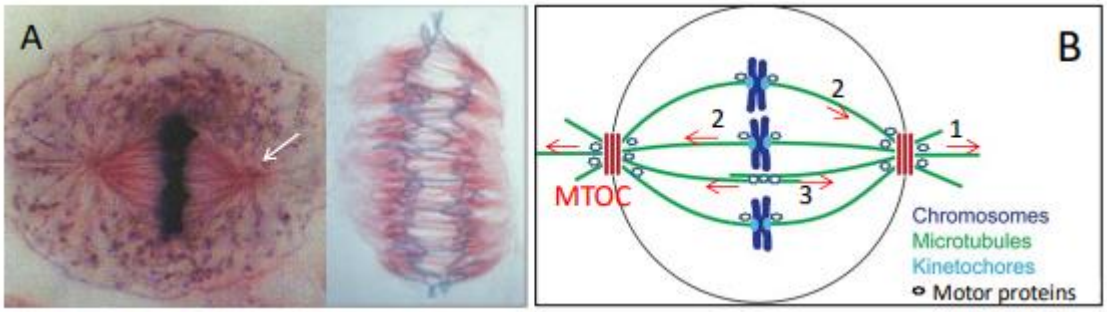


Figure 3. Mitotic division in animal (left) and plant (right) cells.

(A) *Plant cells does not have a centriole (arrow). The separation of chromosomal assemblies is achieved as a result of different types of movements.* ***(B)*** *1. The external (astral) microtubules together with the motor proteins remove from each other the microtubule-organising centres (MTOCs); 2. Kinetochoral microtubules together with a kinesine-13 protein pull the segregated chromatids toward the MTOCs; 3. Antiparallel movement of the polar microtubules. (Source: Lodish* et al.*, 2016)*

Furthermore, the Microtubule-Organising Centres (MTOCs) emerge at the opposite poles of the mitotic spindle, determinating the longitudinal axis for the anaphase movements. After the chromosomes align at the equatorial plate during metaphase, and microtubules attach to the kinetochore-complexes, two different kinetic mechanisms drive the sister-chromatids to segregate and move towards the oppositional poles of the cytoplasm in anaphase.

The two kinetic mechanisms are as follows: i.) Extension and shortening: by the rate of the dynamical assembly and demolition of microtubule subunits at the oppositional ends, the MTOCs' moves toward the poles. ii.) Bidirectional motion effect: plant-specific motor proteins work adherently on the microtubules, so that the concurrent distancing and approximation mechanisms are realised at a few different locations on the spindle strands. Depending on the location of the motor proteins, the following tensile impacts arises: 1) Astral movements: motor proteins work on the outer ('astral') microtubules of the spindle, distancing the two MTOCs by plant specific kinesin proteins (to substitute for the lack of animal dyneines). 2) Kinesine-13 motor protein work on the Kinetochore-attached microtubules (K-microtubules), and atracts the chromatids toward the MTOCs. 3) The polar microtubule complex, a 'kinesine-5' protein-complex works on the polar microtubules, and accomplish the antiparallel distancing ('thrusting') of the MTOCs (McKinley and Cheeseman, 2016).

3.3 The centromere paradox: what defines centromere identity?

Decades of observations confirmed the highly conserved centromeric structure among the eukaryotes and proved its universal and essential role in the faithful distribution of genetic material during mitosis and meiosis (McKinley and Cheeseman, 2016). Early

findings had already revealed the high variation of centromeres in size, formation and structure in a broad range of organisms (Henikoff *et al.*, 2001; Malik *et al.*, 2002; Sanyal *et al.*, 2004; Fukagawa and Earnshaw, 2014) (see section 5.2.2). Discernibly uniform patterns of the unique repetitive centromeric DNA were presumed to be the determinant for the specialised function and suggested to be responsible for the centromere identity (Gent *et al.*, 2011). DNA sequencing discovered the proper centromeric nucleotide sequence arrays in many animal and plant species; their phylogenetic alignments showed an unexpected and extreme variability across different, and even closely related taxa, despite the strictly conserved function. This contradiction between the conserved function and the rapidly evolving shape, size and DNA sequence is referred to as the 'Centromere Paradox' (Montefalcone *et al.*, 1999; Henikoff *et al.*, 2001; Ventura *et al.*, 2001; Gambogi and Black, 2019).

Further, many examples demonstrated that distinct DNA sequences, called neocentromeres, can bind CENH3 and serve as sites for kinetochore recruitment, despite their localisation outside the centromeric region (Henikoff *et al.*, 2001; Jiang *et al.*, 2003; Lamb and Birchler, 2003; Amor *et al.*, 2004; Wang *et al.*, 2014; Liu *et al.*, 2015; Balzano and Giunta, 2020; Robledillo *et al.*, 2020). The exact origins of this dynamic feature are just getting to be known (see below) and unravelling the details of the plasticity of the genome (Tan *et al.*, 2015; Balzano and Giunta, 2020). Scientific studies confirmed that the actual centromeric nucleotide sequence pattern alone is neither sufficient nor necessary to determine the centromeric function (Schuh *et al.*, 2007; Masonbrink *et al.*, 2014; Gambogi and Black, 2019).

The question thus arises how does then the centromeric DNA sequence determine the centromere identity and contribute to the kinetochore formation? Several studies revealed that the centromeric speciation is under epigenetic regulation, i.e., covalent modifications of DNA/RNA by methylation and of histone proteins post-translationally (Sullivan *et al.*, 2001; Cleveland *et al.*, 2003; Hoffmann *et al.*, 2016). As a universal constituent of the centromeric region (see section 2.2.2), the CENH3 protein proved to be a decisive element for centromere identity and as such the logical target for epigenetic speciation of the centromere (Sullivan *et al.*, 2001; Mellone and Allshire, 2003). With the CENH3 recruited in the centromere, this epigenetical speciation can be maintained on the same locus in the germline, through generations (Allshire and Karpen, 2008).

Recent experimental data and observations have started to shed more light on the two contrasting components and the essence of the Centromere Paradox. First, the mechanism of the extremely high sequence diversity in the centromeres of even closely related, intraspecific taxa lies, as proposed by (Wlodzimierz *et al.*, 2023), in its two main constituents, the long arrays of short tandem repeats ('satellites') and the high copy retroelements. Both these elements are 'selfish' by nature, i.e., they can expand to reach a high number by (unequal) crossing over and a characteristic 'copy-paste' mechanism, respectively. In fact, they compete with and counteract each other: the retroelements are specialised to invade and 'colonise' centromeres whereas recombination events between the satellite repeats can eliminate the interspersed retroelements during meiosis as well as mitosis. In fact, it is this constant 'battle' that results in the immense centromeric sequence variation and explains the rapid evolutionary changes observed.

So how can the dynamic centromere structure ensure its conserved function? The answer: it is not the primary DNA sequence but its 3D conformation and unusually folded nucleosomal structure that is essential for the binding of CENH3 and the recruiting of epigenetic factors. New spatial models indicate that the centromeric DNA appears to form noncanonical (non-B-form) 3D structures and (strikingly) more likely so in polyploids, as revealed at least in oat (Wang *et al.*, 2014). The various forms of noncanonical DNA structures were characteristically associated with CENH3-binding regions as well as with centromeric retroelements (Li *et al.*, 2013) and thus may generally be involved in the definition of centromere identity (Kasinathan and Henikoff, 2018). Importantly and confirming this notion, the centromeric region is known to take an unusual, right-handed tetrameric nucleosome structure (instead of the conventional left-handed octameric one) in higher organisms possessing complex centromeres with repeat satellites (Henikoff and Furuyama, 2012). The basis of this nucleosome arrangement is related to the higher-order repeat structure of the satellite DNA.

In conclusion, the specific repetitive and 'selfish' structure of the centromeric DNA appears to be reflected in the unique spatial organisation of the centromeric nucleosomes, which – together with CENH3 binding – defines the boundaries and the function of the centromere (Talbert and Henikoff, 2020).

3.4 The barley and wheat centromere

Besides long arrays of tandem repeated short sequences (‘satellite DNA’) and common canonical terminal repeat-containing motifs (‘retroelements’), the centromeric regions of barley and wheat present considerable structural differences.

The barley centromere includes the G+C-rich (AGGGAG)n satellite sequence (Hudakova *et al.*, 2001) and the high-copy number 7-kb *cereba* element, which is a *Ty3/gypsy*-like retrotransposon (Aragón-Alcaide *et al.*, 1996; Jiang *et al.*, 1996; Presting *et al.*, 1998). Both elements bind CENH3, indicating that they are constituents of the active centromere.

The wheat centromeric region does not carry the G+C-rich satellite DNA, but possesses arrays of other satellite repeats and a centromeric retrotransposon (CRW), which is homologous to the barley *cereba* element (Zhang *et al.*, 2004; Li *et al.*, 2013; Zhao *et al.*, 2023). Some of the short centromeric satellite motives have, however, lost the capacity to bind CENH3 (Kishii *et al.*, 2001; Su *et al.*, 2019). The centromeric region of wheat (and its diploid ancestors) also contains a distinct and specific high-copy retroelement called *Quinta*, which appears to be younger on an evolutionary scale (Li *et al.*, 2013). *Quinta* binds CENH3 at a higher affinity than the more abundant CRW (Li *et al.*, 2013), indicating that *Quinta* and/or CRW may be required for proper centromere function (Houben *et al.*, 2007).

These remarkable structural differences in the centromere organisation also raise the possibility of new or hybrid centromere formation in the genomes of wheat × barley hybrids, which might influence the movement and the fate of particular parental chromosomes (Guo *et al.*, 2016).

3.5 Interspecific wheat hybridisation

3.5.1 The *Triticeae* tribe

The *Triticeae* tribe is economically one of the most important member of the *Poaceae* family. It comprehends approximately 350-500 annual and perennial species including the highly significant cereal crops: bread wheat (*Triticum aestivum* L.), durum wheat (*Triticum turgidum* ssp. *durum* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and triticale (× *Triticosecale* sp. Wittmack ex A. Camus). More than 46% of the species within the *Triticeae* tribe are allopolyploids derived from ancient 2*n*=2*x*=14 diploid progenitors. As a result of many spontaneous hybridisation events the tribe

evolved to contain a wide range of species with different ploidy levels (Al-Saghir, 2016).

Besides the generally present open pollinating feature in many members of the *Triticeae* tribe (Yuan *et al.*, 2015) the second indispensable condition for allopolyploidisation is the existence of the two parents with homologous, homoeologous or somewhat related genetic background. In this case, homology refers to a high similarity of the parental genomes so that chromosome pairing can occur between the two parental genotypes during sexual reproduction. Species within the family which can be sexually crossed with wheat represent a diverse gene pool, which can be used to enhance genetic diversity during wheat improvement (Polgári *et al.*, 2014; King *et al.*, 2017; Grewal *et al.*, 2018; Cseh *et al.*, 2019). Depending on the degree and nature of genetic similarity or diversity between the genomes of wheat and that of the related species, three groups of gene pools can be distinguished:

1. Primary gene pool: consist species carrying genomes homologous to wheat e.g., *T. spelta* (AABBDD), *T. turgidum* (AABB), *T. monococcum* (AA), and *Aegilops tauschii* (DD).
2. Secondary gene pool: at least one genome of the related species is homologous to one of the wheat's genomes, e.g., *T. timopheevi* (AAGG), *T. zhukovskyi* (AAAAGG), *Ae. speltoides* (BB).
3. Tertiary gene pool: No homologous genome is shared between the related species and wheat e.g., *Secale cereale* (RR), *Hordeum vulgare* (HH), *Thinopyrum elongatum* (EE), *Th. intermedium* (JJEESS), *Elymus* spp. (SSHHYY). The frequency of the obtained hybrids is low (Linde-Laursen *et al.*, 2004; Bertin *et al.*, 2009; Levy and Feldman, 2022).

3.5.2 The development and utilisation of wide hybrids

In order to obtain self-fertile progenies following crosses between the wheat and one of the species of the tertiary gene pool the obtained hybrids (wide hybrids) need to undergo one round of genome duplication and/or several round of backcrossings (Figure 4). Genome-duplication resulting in amphidiploid plants may occur spontaneously (Sepsi *et al.*, 2008) or can be achieved via chemical treatment (such as colchicine or caffeine) that cause cell cycle arrest (Nemeth *et al.*, 2015). Several consecutive backcrosses (BC) of the F1 hybrids or the amphidiploids with wheat followed by selfing can lead to the selection of alien chromosome addition lines

(Szakács and Molnár-Láng, 2007; Szakács and Molnár-Láng, 2010; Molnár-Láng *et al.*, 2012) (Figure 4).

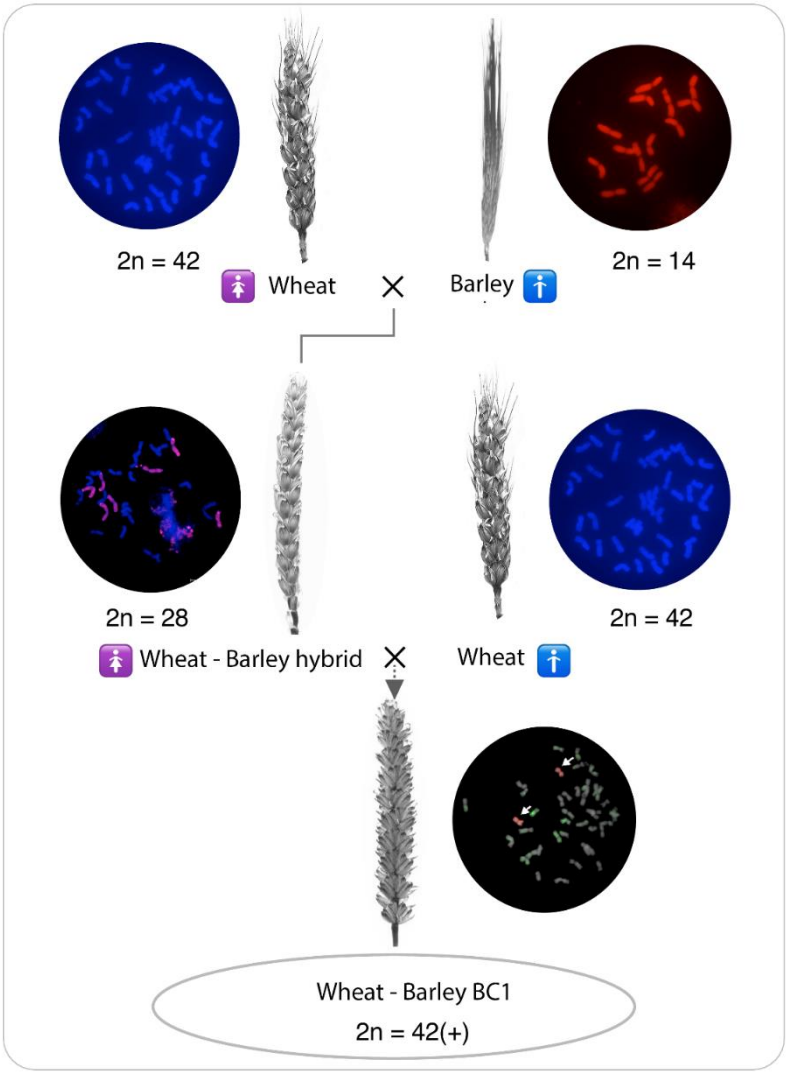


Figure 4. Simplified scheme of wheat × barley hybrid production.

Fertilisation of the hexaploid bread wheat (blue) with diploid barley (red) (upper row) results in the primary F1 hybrids (middle row, left panel). Since this hybrid is self-sterile multiple backcrosses (middle row) will ultimately produce novel wheat genotypes (bottom row) containing barley chromosomes or chromosome segments s *(arrows). (Source: Sepsi et al., 2020)*

These addition lines are further backcrossed and selected for recombinant lines that contain smaller chromosome segments or translocations carrying the gene(s) of interest, e.g., resistance to biotic or abiotic stress (Molnár-Láng *et al.*, 2000; Cseh *et al.*, 2013; Kruppa *et al.*, 2013; Danilova *et al.*, 2018). After several years of evaluation the best prebreeding lines can finally be used as crossing partners with recipient commercial cultivars.

The transmission of the foreign (or alien) genes traditionally (by non-GM transfer) in

the scope of the tertiary gene pool in cereals was historically achieved from diploid rye, which led to the first man-made and successful new species, triticale (× *Triticosecale*). As a supporting fact, the present *Triticale* cultivars combine the high yield of wheat with the adaptatability of rye to abiotic stresses and fungal pathogens (cereal rusts and powdery mildew) allowing its cultivation in a larger area than ever before (McGoverin *et al.*, 2011).

3.5.3 Wheat × barley hybrids: results and limitations

The idea of generating a hybrid between the two most important small-grain cereals, wheat, and barley, goes back to 120 years. The first anecdotic report about a new wheat × barley hybrid plant was related to William Farrer, a pioneering Australian breeder, and was dated to 1904. The first documented attempts to produce the hybrid in either combinations (wheat (♀) × barley (♂), barley (♀) × wheat (♂)) were reported by the Danish scientist Anton Kruse in the first half of the 1970s (cit. Shepherd and Islam, 1981). During the next decades, the main efforts were focused on the identification of efficient parental genotype-combinations, but these were met with limited success. The F1 hybrids were completely self- and male-sterile, their genome duplication never worked out and backcrosses with recurrent parents was very inefficient.

For crossings in the direction of barley (♀) × wheat (♂), four barley and seven tetraploid or hexaploid wheat genotypes were tested first in a total of 25 combinations and only one, ‘Betzes’ × ‘Chinese Spring’ yielded a high frequency, 15.4% (pseudo)seed set but the number of obtained embryos and plants remained unknown (cit. Shepherd and Islam, 1981). It took almost a decade until it became generally recognised and accepted that – despite the tempting pollination success – this crossing direction is a dead end. The main reason for the failures was partial pistillody, i.e., the transformation of one or more stamens in a variable number of florets to pistil-like structures, which was sporadic but general among the different barley × wheat F1 hybrids whereas it did not occur in other *Hordeum* sp. × wheat hybrids (Islam and Shepherd, 1990). In addition, the incidence of pistillody and self-sterility progressively increased in successive BC1 and BC2 generations parallel with the increasing proportion of the wheat genome and chromosomes in the progeny. Taken together, these observations pointed to a specific incompatibility between the *H. vulgare* cytoplasm and the wheat genome as the reason behind pistillody. It was time to test the presence of pistillody in reciprocal crosses.

The first viable hybrid plants in the opposite direction of hexaploid wheat (♀) × barley (♂) were produced by the very end of the 1970s. Out of six combinations between three wheat and three barley cultivars, Islam *et al.* (1981) found that only one ('Chinese Spring' × 'Betzes') resulted in 0.6% of hybrid embryos and plants from more than 3,000 pollinated florets – about 10 times less than in the other crossing direction. While pistillody has never been observed, limited progress has since been achieved especially in extending the range of highly compatible genotypes as summarised below based on Polgári *et al*. (2014). For instance, only two out of eight combinations between two wheat and six barley cultivars produced more than 1% of regenerated plants (Finch and Bennett, 1982). Similarly, the testing of 12 combinations between five wheat and seven barley cultivars revealed just four combinations with more than 1% of hybrid embryo induction and plant regeneration (Wojciechowska and Pudelska, 1993). When (Koba *et al.*, 1991) analysed all the 16 combinations between four wheat and four barley cultivars, a mere seven combinations (primarily involving 'Shinchunaga' wheat and 'Betzes' barley) produced more than 1% of hybrid embryos. The same two cultivars made a major contribution to seven out of 11 combinations of four wheat and three cultivated barley genotypes that resulted in at least 1% of plant regeneration (Taketa *et al.*, 1995). Finally, out of the 14 winter wheat × winter barley combinations tested by Molnár-Láng *et al*. (2000) only three produced viable hybrids at a very low frequency.

Studying the role of the wheat background in hybrid success rates, crossing 23 spring wheat cultivars with the standard 'Betzes' barley identifed about half of them producing more than 1% and only four (17%) above 10% of hybrid embryos (KOBA and SHIMADA, 1992). To test the opposite side, Taketa *et al.* (1998) crossed 'Shinchunaga' wheat as a standard with 30 barley cultivars and found that at least 23 of them (77 %) produced more than 1% of hybrid embryos, and 14 combinations (47%) resulted in more than 10%. Based on these two observations, the bottleneck in finding genotypes with high crossability in wheat × barley hybridisation appears to be more on the wheat side.

After eliminating the overlaps in the above data, it can be concluded that altogether about 100 combinations between 29 wheat cultivars and about 55 barley accessions have been tested until recently and about 45 combinations produced 1% of hybrid embryos, but only 20 of them yielded more than 10%. In addition, in very few of the highly responding combinations were cultivars with a significant agronomic value

involved. Therefore, there is a real need to find more responsive combinations, preferably with useful genotypes.

Based on the above considerations, a crossing programme was initiated in Martonvásár which led to the hitherto most efficient wheat × barley hybridisation system (Polgári *et al.*, 2014) after testing only three new parental genotype combinations. A particularly high, 14% frequency of plant regeneration per pollinated florets was achieved in the genotype combination of 'Sichuan' wheat × 'Morex' barley, which was a leading malting cultivar. The genome composition and hybrid nature was confirmed in 12 out of 40 regenerated plants by ploidy analysis, GISH and the application of chromosome arm-specific molecular markers (SSR and STS). Later on, this high efficiency was reproduced with the same parental combination but this time in a population of 200 plants (Polgári *et al.*, 2019).

These high numbers of F1 hybrids are required as the starting material for further utilisation including the generation of fertile amphiploids, translocation lines and other chromosome engineering products (Figure 4) followed by eventual application for the genetic improvement of wheat and barley.

3.5.4 Uniparental chromosome elimination in interspecific hybrids

The primary cause for the low efficiency (in the order of a few percentages) of wheat × barley hybridisation is the lack of highly crossable parental genotype combinations (see section 2.5.3). Other bottlenecks include the need for healthy plant material, optimal and controlled environment to ensure viable pollen, and efficient *in vitro* plant regeneration from embryos rescued from the endosperm-less pseudoseeds. While many of these factors can be optimised or kept under control, even the most efficient hybrid technology faces an ultimate challenge – the elimination of a partial or the complete chromosome complement of one (usually the pollen donor) parent in the developing hybrid plant. The process of this uniparental genome elimination can occur during the early vegetative (postzygotic) development and/or the reproductive (meiotic) phases. While the latter phase is well known and associated with the lack of homoeologous pairing between the parental chromosomes during meiosis, the preceding early mitotic elimination step – despite its prevalence – is much less characterised. Therefore, while excellent reviews have already been published (Houben *et al.*, 2011; Ishii *et al.*, 2016; Comai and Tan, 2019), it is briefly summarised here, based on Polgári *et al*. (2019).

Uniparental chromosome elimination in cereals

The uniparental chromosome elimination is particularly well described in hybrids obtained between members the botanical tribe of *Triticeae* (e.g., cereals), where it is primarily, though not exclusively, confined to the paternal genome donor (Houben *et al.*, 2011; Zhao *et al.*, 2013). A closer analysis of a recent literature survey on parental genome elimination (Ishii *et al.*, 2016) revealed that 65 intergeneric or interspecific hybrid combinations contained one or both parents from within the *Triticeae* tribe. Of these 65 hybrid combinations, the overwhelming majority (94% or 61 hybrid types) lost, partially or completely, the paternal or male genome, and only four types (6%) featured elimination from the maternal genome. Exclusively haploids, i.e., the complete loss of the maternal (three hybrids, 5%) or the paternal (40 hybrids, 61%) genome, were found in 43 (66%) hybrid combinations. Another five combinations (8%) possessed mixed scenarios: they contained no (maternal haploids), the partial or the full paternal genome: the only intergeneric combination of them was hexaploid wheat × cultivated barley (*H. vulgare*), the other four combinations being interspecific *Hordeum* hybrids again with *H. vulgare* as the paternal partner. All these 48 (43+5) combinations (74%) required *in vitro* embryo rescue to generate viable plants. The remaining 17 hybrid types (26%) also had mixed outcomes: full, but no partial, hybrids as well as haploids (one paternal and the rest maternal).

It is clear from the above analysis that our focus, the wheat × barley has a special place in the kingdom of hybrids as it is the only intergeneric combination capable of producing an extreme range of outcomes, from haploids to full hybrids.

The elimination process in cereals

The precise mechanism of chromosome elimination is complex and appears to be species- or even case-specific. Besides the asynchronous cell cycle or other imbalances between the parental genomes (Subrahmanyam and Kasha, 1973; Bennett *et al.*, 1976), alternative mechanisms have also been proposed such as no or markedly different attachment of the two parental chromosome sets to microtubuli during early mitotic divisions in wheat × maize zygotes (Mochida *et al.*, 2004). In other cases, chromosome elimination during the embryogenesis of cultivated barley (*H. vulgare*) × *H. bulbosum* hybrids was associated with the formation of micronuclei, abnormally condensed chromatin, and chromosome fragments (Gernand *et al.*, 2006). These rearranged chromosomes and micronuclei derived from the breakage of bridges and retention of

acentric fragments in anaphase, respectively. Thus, chromosome elimination is not always due to the malfunction of the kinetochores binding to the microtubuli but also to the failure of the sister chromatids to segregate at anaphase (Ishii *et al.*, 2010).

The well-known spatial separation of parental genomes within the nucleus of interpsecific cereal hybrids both in the interphase (Schwarzacher *et al.*, 1989; Gernand *et al.*, 2005) and/or during mitoses (Leitch *et al.*, 1991; Mochida *et al.*, 2004) can also be a trigger for genome elimination. Indeed, in wheat × pearl millet hybrids, the paternal chromatin destined for elimination was first positioned at the nuclear periphery during the interphase, followed by a structural reorganisation of the paternal chromosomes during mitosis, which then led to nuclear extrusions and formation of micronuclei (Goshima *et al.*, 2003; Gernand *et al.*, 2005). In contrast, in oat × pearl millet hybrids, in which the pearl millet genome is stably maintained, no peripheral positioning, nor the chromosome rearrangements or formation of micronuclei were observed (Ishii *et al.*, 2010).

More recently, uniparental centromere inactivation was proposed as the cause of paternal chromosome elimination in wheat or barley × *H. bulbosum* hybrids (Sanei *et al.*, 2011; see below, p. 27). Clearly, different mechanisms of chromosome elimination may apply case-by-case in interspecific cereal hybrids, depending on the parental species involved.

As to the temporal dimension of the process, the uniparental elimination of chromosomes is usually completed in the early embryo during a few initial cell divisions within a time window of 5-8 DAP (days after pollination) in *H. vulgare* × *H. bulbosum* hybrids (Subrahmanyam and Kasha, 1973; Bennett *et al.*, 1976; Gernand *et al.*, 2006), 3-4 DAP in wheat × maize (Laurie and Bennett, 1989), 1-2 DAP in wheat × *Imperata cylindrica* (Komeda *et al.*, 2007), and 8-12 DAP in wheat × pearl millet crosses (Gernand *et al.*, 2005). Smaller groups of one-three chromosomes but up to all seven chromosomes can be eliminated in a single cell cycle (even during the first mitosis!), both in the embryonic and endosperm tissue of *H. vulgare* × *H. bulbosum* hybrids (Bennett *et al.*, 1976).

Are chromosomes eliminated preferentially or in a random order and manner?

Previous research reported on the preferential, biased elimination of particular barley chromosomes in wheat × barley hybrids (Koba *et al.*, 1991; Taketa *et al.*, 1995). These authors concluded that the barley chromosomes 1H and 5H (Koba *et al.*, 1991) or 4H

and 5H (Taketa *et al*., 1995) were preferentially lost in the analysed 13 and 19 partial hybrid (hypoploid) plants, respectively. Remarkably, chromosome 4H was, contrary to the conclusion of Taketa *et al*. (1995), the last one to become eliminated according to Koba *et al*. (1991).

In contrast, in what could be considered as the first systematic and large-scale analysis on uniparental chromosome elimination in intergeneric hybrids of cereals or any plant, a large population, 210 F1 plants from two wheat (♀) × barley (♂) cross combinations were analysed by barley chromosome arm-specific DNA markers and statistically for the frequency and pattern of paternal chromosome elimination by mitosis during early vegetative development (Polgári *et al*., 2019). The analysis of the data revealed no preference in the elimination of individual barley chromosomes from the hybrids, so the initial genome elimination process (at least in this cross combination) can be considered as random.

The obvious explanation for the contradiction in the above studies could be that the different genotype combinations tested, and the diverse evaluation techniques used preclude direct comparisons and the drawing of reliable conclusions.

Yet, two kinds of explanations may account for the discrepancy between these three studies. The first element is the size of the experiments: Koba *et al*. (1991) and Taketa *et al*. (1995) determined the chromosome constitution (by C-banding and isoenzyme markers) in 19 and 33 plants, respectively, compared to the 210 plants analysed by molecular markers (Polgári *et al*., 2019). Obviously, the size and the statistical power of the first two studies were too small to reliably evaluate the elimination pattern of individual chromosomes.

The second explanation is related to an often-neglected advantage of molecular markers over the cytogenetic monitoring of chromosome composition in interspecific hybrids. Cytogenetic analysis is performed on individual cells and can identify multiple cells with different chromosome numbers and composition within the same tissue sample, which hampers the precise identification of the corresponding, cytogenetically mosaic plant. Indeed, Koba *et al*. (1991) and Taketa *et al*. (1995) found and excluded 13 and 18 such mosaic plants, respectively, which significantly weakened the power of their data. The DNA template, however, is purified from tissues composed of thousands of cells, therefore the rare mosaic variants are masked

or underrepresented in the final DNA. Also, the exponential nature of PCR further diminishes the detection of these variants.

In summary and based on the above evaluation, it appears that the early (mitotic) uniparental chromosome elimination could be a random process. It remains to be seen whether the same is true for the later, meiotic phase.

The role of CENH3 histone proteins in chromosome elimination

Sanei *et al.* (2011) produced both stable and unstable hybrids of *H. vulgare* × *H. bulbosum*, in order to identify the initiating factors of the uniparental chromosome elimination. Series of crossings were made with *H. vulgare* 'Emir' as a female partner, and *H. bulbosum*, as a pollen donor. The pollinated mother plants were then divided into two groups, and further incubated at two different temperatures in order to control the chromosome elimination process after pollination (Sanei *et al.*, 2011). An earlier article reported that temperatures above 18°C after pollination support chromosome elimination, whereas temperatures below 18°C promote the retention of the parental chromosomes (Pickering, 1985).

The uniparental elimination of chromosomes in unstable hybrids was accompanied by the loss of CENH3 fom the centromeres. The centromere inactivity of chromosomes in *H. vulgare* × *H. bulbosum* hybrid embryos appeared to trigger the mitosis dependent process of uniparental chromosome elimination, where exclusively *H. bulbosum* chromosomes were eliminated. GISH combined with immunostaining demonstrated that chromosome failure was strongly associated with the insufficient level of CENH3 recruitment into the hybrid centromeres. Not all centromeres incorporated CENH3 proteins in the instable hybrids, in contrast to the stable hybrids, that recruited CENH3.

Contrary to other proteins, whose expressions mainly occur during the S phase, CENH3 protein expression is almost continuous during the whole cell cycle, although at a lower intensity. CENH3 resources are not required to be renewed (newly expressed) during each cell cycle of regular embryogenesis, as one part of the CENH3 proteins are transferred into the daughter cells. As dynamic centromeric events accelerate in G2 phase, the chromatin condensates together with CENH3 loading up to metaphase. In early embryogenesis of hybrid plants. the duration of G2 phase may not be sufficient for the appropriate recruition of the CENH3 protein ino the centromeres, and this insufficient loading may result in inactive centromeres prone to elimination (Chen *et al.*, 2015). Centromeric loss of CENH3 protein, rather than

uniparental silencing of *CENH3* genes causes the centromeric inactivity. In a stable species combination, the cross-species incorporation of CENH3 occurs despite centromere-sequence differences. Not all CENH3 variants are incorporated if multiple CENH3s are present (coexisting) in species combinations. The lack of cross-species incorporation might impose a barrier in the way of hybridisation.

Hordeum bulbosum chromosomes are eliminated several days after pollination (till 3-6 DAP) independent to the crossing direction. Even so, hybrids containing both sets of parental chromosomes can be obtained (Humphreys, 1978). Elimination depends on genetic factors and the temperature after fertilisation (Pickering, 1985).

The chromosome elimination is such an effective and reliable process, that ultrawide crossings are regularly used as a method for haploid induction. Using chemical genome duplication, doubled haploid or amphiploid homozygous germlines can be obtained with a restored fertility.

Several studies have been carried out with the purpose to promote breeding by benefiting from the haploid induction technology. Double haploidy achieved within one generation implies the useful consequences via homosigosity present at all genomic loci. There is agreement that proper chromosomal localisation of the CENH3 protein is crucial, for ensuring the assembly site for the kinetochore-complex of active centromeres. Any disfunctionality in tanscription, translation, or spatial conformation of CENH3 may negatively affect segregation, resulting centromere inactivity. Karimi-Ashtiyani and collegues have used ‘Golden Promise’, sugar beet, and *Arabidopsis* lines carrying a single point amino acid mutation in the centromere-targeting domain of the CENH3 protein. Results in barley revealed that a such minor change can lead to a reduced CENH3 loading when crossing a mutant with a wild type (hybrid genome includes mutant and wild type CENH3 at the same time) (Karimi-Ashtiyani *et al.*, 2015). The phenonomenon of compensation may happen in other cases of wheat × barley crossings, as two genotype-specific *CENH3* genes and proteins are present in wheat barley hybrids. This biological phenomenon, the competition may explain the eliminating process in the produced primary hybrids.

Uniparental elimination may represent a protective mechanism during sexual hybridisation between unusually distant partners, by preserving genome integrity following the „genome shock” (McClintock, 1984). The precise mechanism is complex and appears to be species-specific. On the other hand, it seems that

chromosome elimination is a phenomenon that in a certain way determines crossing boundaries between the species involved, so ultimately limits the success of ultrawide hybridisation. Uncovering and modifying the underlying mechanisms of chromosome elimination a wider range of intergeneric crossings could be accessible for breeding programmes, generated via non-GM-mediated gene transfer.

3.6 Cytological methods to analyse the DNA and protein components of the chromatin in hybrids

3.6.1 In situ *hybridisation*

In situ hybridisation (ISH) is a cytological technique used to localise specific DNA or RNA sequences on fixed chromosome/nuclei preparations or tissue sections. The method takes advantage of the process of DNA denaturation at high temperature resulting in the separation of the DNA double strands that form the chromosomes on the cytological preparation. Re-naturation follows the rules of complementarity and occurs when lowering the temperature. At this stage, a labelled and denatured (single strand) DNA (the probe) is added to the cytological preparation, so that the target sequence becomes complemented by the labelled DNA probe. The signal produced by the probe can then be visualised by microscopy. The i*n situ* hybridisation technique was originally developed in the late sixties by using isotopic probes detected by autoradiography (Pardue and Gall, 1969; John *et al.*, 1969). Autoradiographic detection is however time consuming (up to four days), results in poor resolution and instead of determining sequence localisation within a single cell, it requires statistical methods of a high number of cells. These limitations were overcome by the introduction of nonradioisotopic methods that were adapted to plants. Rayburn and Gill (1985) used a biotinylated pSc 119 probe detected by an enzymatic reporter molecule (horseradish peroxidase) to construct a molecular karyotype allowing the identification of the B genome chromosomes wheat. Out of the B-genome, the pSc119 probe reliably identified the chromosomes 4A, 2D, 3D and 5D. Fluorescence *in situ* hybridisation is another nonradioisotopic method that takes advantage of fluorochrome-labelled probes to generate a signal within the cytological preparations (Bauman *et al.*, 1980; Bauman *et al.*, 1981; Langer-Safer *et al.*, 1982). Using this technique different fluorochromes can be used to label multiple DNA probes, allowing the simultaneous mapping of two or more target sequences. FISH is a powerful technique to physically localise repetitive DNA sequences (Maluszynska and Heslop-Harrison, 1991; Mukai *et al.*, 1993) multicopy gene families (Mukai *et al.*, 1990;

Leitch and Heslop-Harrison, 1992; Lapitan *et al.*, 2011) and low- or single-copy genes (Ambros *et al.*, 1986; Simpson *et al.*, 1988).

Genomic *in situ* hybridisation (GISH) allows the visualisation of whole genomes in hybrids and allopolyploid taxa. GISH uses total genomic DNA extracted from the parental (in the case of hybrids) or ancient progenitor species (in the case of allopolyploids) as a probe in order to detect the targeted chromosome complement on mitotic chromomes (Schwarzacher *et al.*, 1989). Introgressions of chromosome segments adding agronomically useful genes to a crop species can be followed in detail, revealing the size of the introduced fragments and localisation along the host chromosome (Mukai *et al.*, 1993).

3.6.2 *Immunohistochemistry*

Immunohistochemistry (also called immunolabelling) uses antibodies to identify sites of protein antigens in biological samples (tissue sections). When the method is applied to nuclei- or chromosome preparations it is referred to as immunocytochemistry (ICC). The visualisation of the antibodies bound to their antigens (termed as primary antibodies) is accomplished by using a second antibody (secondary antibody) which is attached to a label (e.g., a fluorophore). The generated signal can be detected by microscopy techniques. The first immunohistochemical study was published by Coons *et al*. (1941) who used fluorescein isothiocyanate (FITC)-labeled antibodies to detect pneumococcal antigens in infected tissues. Other frequently used immunohistochemistry reporters include enzyme labels e.g. peroxidase (Nakane and Pierce, 1966), alkaline phosphatase (Mason and Sammons, 1978) and colloidal gold (Faulk and Taylor, 1971) label which have been used for light and electron microscopy. The advantage of immunohistochemistry over the traditional protein staining technique that it has zhe ability to detect selected proteins or protein families within tissues or single cells. Additionally, the non denaturing fixation and preparation technique allows the visualisation of target proteins within the 3D structure of the biological material.

3.6.3 *Standard methodology for nuclei preparations suitable for immunoFISH*

Combination of the two techniques above is suitable to study selected DNA sequences and proteins within a single cell, opening the possibility to understand complex cell biology processes at a detail that cannot be achieved by other methods. Technically, both *in situ* hybridisation and immunolabelling require contrasting fixation and preparation methods, which makes their combination highly challenging. For instance,

in situ hybridisation uses a denaturing tissue fixation and cell preparation in order to ensure the accessibility of the target DNA sequences while ICC demands a non-denaturing fixation to preserve protein antigens.

Standard procedures of plant nuclei preparations for combined ICC and FISH (i.e., immunoFISH) follow a relatively lengthy non-denaturing fixation (approx. one hour) to preserve nuclear proteins and general ultrastructure. Plant cell wall removal is ensured by an enzymatic treatment (e.g. cytohelicase, pectinase, pectolyase, cellulose, or their mixtures) and manual tissue maceration, which would allow the successful release of preferably cytoplasm-free cells on the surface of microscope slides (Pavlova *et al.*, 2010; Pasternak *et al.*, 2015; Bey *et al.*, 2018).

The effectiveness of this procedure largely depends on enzyme quality, which can vary among the manufacturers (or even batches of the same manufacturer). Standardisation of cell wall removal by enzyme digestion is thus highly challenging especially as enzyme activities significantly decrease with time due to repeated applications and multiple freeze-thaw cycles. Preparation of plant nuclei for microscopic observation requires meticulous training and a great deal of skills for the effective manual release of cells (by needles, brass rods, or tweezers) from intact tissues onto the microscope slides.

4 HYPOTHESIS AND OBJECTIVES OF THE THESIS

Both parental (wheat and barley) CENH3 proteins are hypothetised to play a role in chromosome elimination or retention. The aim of the present study was to better understand the chromosome elimination process in wheat × barley primary hybrids, and the role of parental CENH3 variant incorporation into the centromeric DNA. We thus analysed chromosome stability in primary hybrids along with the capacity of the centromeric DNAs – originating from each parents (i.e., wheat and barley) but residing into the same cell nucleus – to load con-species and cross-species CENH3 proteins. For that purpose, wheat and barley centromeres needed to be differentiated from each other as located in the nucleus.

The following objectives were explored in the course of the present work:

1. This study aimed to design and implement an effective research tool allowing the high resolution and reliable detection of different CENH3 variants of wheat and barley. This was planned to be realised by species- and variant specific polyclonal antibody design and production. Additionally, the development of a cell nuclei preparation method aimed to facilitate the effective tracking of CENH3 protein variants together with the 3D visualisation and high resolution detection of specific DNA sequences.
2. As a further requirement of our study, new plant materials were planned to be produced in the form of newly produced wheat × barley hybrid plants. The new plant material was aimed to undergo a complete molecular cytological characterisation where the presence of the barley chromosomes and the precise chromosome components (including identification of barley chromosomes) of the hybrid plants could be determined unequivocally.
3. The fully optimised research tool (antibodies and molecular cytology techniques) suitable to study the new plant material was used to perform immunoFISH and laser scanning confocal microscopy to elucidate the incorporation of the parental CENH3 proteins into the wheat and barley centromeric DNAs in the somatic nuclei of F1 hybrids. Wheat and barley centromeric sequences were clearly identified and localised at a single-cell resolution.

4. Ultimately CENH3 incorporation was planned to be deciphered in detail during the early stages of meiotic cell division, reflecting chromosome stability in different CENH3 incorporation scenarios.
5. The outcome of the present research would answer the role of parental CENH3 proteins and their combinations in the chromosome activity, inheritance and behaviour during the mitotic and meiotic cell cycles in wheat × barley F1 hybrids.

5 MATERIALS AND METHODS

5.1 Plant material

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 landrace '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 the 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 photoperiodism (150-500 μmol m^{-2} s^{-1} PPFD).

Two F1 hybrid plants were selected for further cytological analysis (see Results 4.1). Progeny backcrossed (BC1) to the original M1 wheat parent was also generated and analysed.

5.2 Cytological procedures

5.2.1 Simultaneous GISH-FISH procedure

5.2.1.1 Fixation of root tips

Seeds were germinated at room temperature in Petri dishes on moist filter paper. As soon as the seeds were imbibed, they were transferred to a cold-room (+4°C) for at least 72 h to obtain synchronised cell divisions and provide uniform root growth. The seeds were subsequently placed to room temperature for 24 h until the roots reached a length of 1.5-2 cm.

Vigorously growing roots of potted plants and of germinated seeds (see above) were placed into 10 mL glass vials containing ice-cold distilled water with melting ice (1:2 volumes of ice and distilled water). The vials were transferred into a polystyrene box filled with ice, covered with a lid, and placed into a cold-room at 4°C for 24-26 h.

Roots were then fixed in 5 mL glass vials containing Clarke's fixative (3:1 v/v mixture of absolute ethanol and glacial acetic acid). The vials were incubated at 37°C for 5

days that was followed by a two-hour 4% (w/v) acetocarmine (Carmine powder, Sigma-Aldrich, C0521) staining at room temperature. The roots were subsequently immersed in fresh Clarke's fixative and stored at -20°C for at least two weeks before use for chromosome preparations.

5.2.1.2 Chromosome preparations by squashing

The fixed roots were placed into 45% (v/v) acetic acid for 10 min and subsequently placed on Superfrost microscope slides. The root tips were removed with a razor blade and the meristematic cells were pressed out with a 'micro-spade' tool into an 8 µL drop of 45% acetic acid. Any visible debris or plant tissue was discarded with a pair of tungsten needles before placing an 18x18 mm coverslip (Menzel-Gläser) carefully on top of the cell mixture. The slide was subsequently heated over an open flame for a few seconds. When the slide cooled down a slight pressure was applied by using the thumb while avoiding the movement of the coverslip. The quality of the preparations was evaluated under a phase-contrast microscope (Leica DM2000 Led).

5.2.1.3 Simultaneous *in situ* hybridisation

DNA extraction and probe labelling

For preparing the probe for GISH experiments, total DNA of 'Golden Promise' barley was extracted from 5 g of fresh young leaves by using the CTAB method (Rogers and Bendich, 1994). The DNA was then fragmented for 6 min in a pressure cooker to obtain 300-500 bp long DNA fragments. The quality and the length of the DNA fragments was assessed by agarose gel electrophoresis while the concentration of the DNA solution was measured spectrophotometrically (ND-1000UV/Vis, Nanodrop Technologies). One µg of fragmented DNA was directly labelled by nick-translation (AF594 NT Labeling Kit, PP-305L-AF594; Jena Bioscience) and 40-50 ng of the labelled barley DNA probe was applied per prepared sample.

To obtain unlabelled total wheat DNA for blocking unspecific signals, DNA was extracted from fresh young leaves of the M1 parental wheat by the CTAB method (see above).

For the preparation of the FISH probes, the 5S rDNA coding and flanking noncoding region of barley was amplified by PCR as described by Fukui *et al*. (1994) and directly labelled by nick-translation (AF488 NT Labeling Kit, PP-305L-AF488, Jena Bioscience). The barley-specific G+C-rich centromeric satellite sequences were

amplified according to (Hudakova *et al.*, 2001) and directly labelled by nick-translation (AF647 NT Labeling Kit, PP-305L-AF647, Jena Bioscience). A 576-bp fragment from the integrase region of the polyprotein gene of the wheat centromeric retrotransposon (CRW) was amplified by the primers 5'-GTTTGTCCATCAGTTTGG-3' and 5'-GTTTGTCCATCAGTTTGG-3'. The primers were specifically designed to amplify a polymorphic region within the integrase domain of CRW as well as the barley *cereba*. The amplified CRW sequences were labelled by nick-translation (DIG-Nick Translation kit, Roche Diagnostics, 11745816910) and the digoxigenin signals were detected by Anti-Digoxigenin-Rhodamine (Roche Diagnostics, 11207750910).

Preparation of the probe mix

The hybridisation mixture contained 54% (v/v) deionised formamide (Sigma-Aldrich, F9037) and 2.4% (v/v) dextrane sulphate (Sigma-Aldrich, 42867) diluted in saline sodium citrate buffer (2X SSC: 0.3 M NaCl and 30 mM trisodium citrate dihydrate, pH 7.0). The hybridisation mix was stored at -20°C and used together with the labelled probes and the blocking DNA.

The probe mix included 17 μL of the hybridisation mixture, 40-80 ng of each of the labelled probes (1.0-1.2 μL of the GISH probe and 1-2 μL of the FISH probes), and an excess of unlabelled (blocking) wheat total DNA (at a ratio of 1:30) per slide.

Denaturation and hybridisation

The probe mix was denatured at 85°C for 8.5 min in a PCR machine and immediately placed on ice to avoid re-naturation of the DNA strands. A final 22 μL volume of the probe mix was pipetted on each slide and again denatured for an additional 3 min at 75°C in a PCR machine equipped with a stainless-steel plate (Thermal Cycle 2720, Applied Biosystems, Life Technologies). The slides were quickly removed from the hot plate and placed into a slide hybridiser (Grant-Boekel SM-30) for incubation at 37°C overnight.

Post-hybridisation washing

The slides were removed from the slide hybridiser and placed into a plastic jar containing 1X PBS (phosphate-buffered saline: 137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). Following a 2x5 min washing, the preparations

were covered with 24x32 mm glass coverslips (Menzel-Gläser) and mounted with 12 µL of Vectashield Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories Inc.).

5.2.2 Immunolabelling and immunoFISH procedure

5.2.2.1 Paraformaldehyde fixation

Growing roots of potted plants were harvested and placed into 10 mL glass vials containing ice-cold distilled water with melting ice (1:2 volumes of ice and distilled water). The vials were transferred into a polystyrene box filled with ice, covered with a lid, and placed into a cold-room at 4°C for 24-26 h. Root tips were then placed into 4% (w/v) paraformaldehyde (PFA) (16% Formaldehyde Solution, Thermo Scientific, cat. No. 28908) solution for 30 min with the first 5 min under vacuum and washed in 1X PBS containing 0.3% (v/v) Triton X-100 (Sigma-Aldrich, T8787) and 0.3% (v/v) IGEPAL (Sigma-Aldrich, 18896) for 2x5 min. This method was used for antibody testing as well (see section 3.3).

Immature anthers in meiosis were also fixed with 4% PFA as above but supplemented with 0.5% IGEPAL and treated only for 15 min, with the first 5 min under vacuum. Pollen mother cells were slide-mounted with a pair of fine tungsten needles and squeezed into a drop of 1X PBS containing 0.5% IGEPAL.

5.2.2 2 Nuclei preparation by dropping

Five root tips were homogenised in LB01 solution (15 mM Tris-HCl, 2 mM Na2EDTA, 0.5 mM spermine, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, pH 8.0; Doležel *et al*., 1989) in a 2 mL KIMBLE Dounce tissue grinder set (Sigma-Aldrich, D8938). The cell suspension was subsequently filtered through a 70-µm and 40-µm cell strainer (pluriStrainer Mini 70 µm, 43-10070-40 and pluriStrainer Mini 40 µm, 43-10040-40; pluriSelect). The nuclei suspension was centrifuged at 2000 × *g* at 4°C for 5-10 min. The supernatant was discarded, and the pellet was resuspended in 50 µL of 1X PBS containing 0.3% Triton X-100 and 0.3% IGEPAL. Five to eight µL of nuclei suspension were then dropped onto adhesion microscope slides (Epredia Superfrost Plus or Superfrost Ultraplus, Menzel-Gläser) and air dried at room temperature. The preparations were then suitable for immediate immunolabelling, or

after freezing on dry ice, they could be stored at -80°C for several weeks. This method was used for antibody testing as well (see section 5.3).

5.2.2.3 Immunolabelling and combined FISH

Immunolabelling

Fifty µL of 50-100x diluted primary antibody solution (0.5-1 µL in 50 µL of TNBg [0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.5% (w/v) blocking reagent (Sigma-Aldrich, 11096176001) with 0.3 M glycine (Sigma-Aldrich, G8898)] containing 0.2% Triton X-100, 0.2% IGEPAL, and 0.01% (v/v) Saponin (Sigma-Aldrich, 47036) were applied per slide, then covered by a plastic foil and incubated at 37°C in a humid chamber for 1 h, then at 4°C for overnight, and incubated again at 37°C for 1 h. After washing in 1X PBS for 2x5 min, 50 µL of 200x-diluted secondary antibody solution (0.24 µL in 50 µL of TNBg) were applied and incubated at 37°C in a humid chamber for 1 h. The following secondary antibodies were used in the present work: abberior STAR RED, goat anti-guinea pig IgG (Abberior, Göttingen, Germany, STRED-1006), abberior STAR RED, goat anti-rat IgG (STRED-1007), abberior STAR RED, donkey anti-sheep (STRED-1056), abberior STAR RED, goat anti-rabbit IgG (STRED-1002). After washing in 1X PBS for 2x5 min, images were taken after mounting (Vectashield Antifade Mounting Medium with DAPI). This method was used for antibody testing as well (see section 7.3).

Prehybridisation treatments: permeabilisation and post-fixation

To ensure the FISH probes' access to the chromatin fibres, an additional nucleic membrane permeabilisation was needed. The slides were immersed into a glass jar filled with 0.3% (w/v) CHAPS hydrate surfactant (Sigma-Aldrich, C3023), 0.3% Triton X-100, and 0.3% IGEPAL in 1X PBS and incubated at room temperature for 15 min. The slides were then transferred to 1X PBS and washed for 5 min and for 10 min. The specimens were digested by 50 µL per slide of fresh 50 µg/mL pepsin (Roche Diagnostics, 10108057001) solution containing 1 mM HCl. Enzymatic digestion was performed at 37°C in a slide hybridiser (Grant-Boekel SM-30) for 1-2 min depending on the quality of the slide. After washing in 1X PBS for 2x5 min, post-fixation by 50

µL per slide of 37°C preheated 4% PFA followed, the slides were covered with plastic foil, and incubated at room remperature for 5-10 min. Finally, the slides were washed in 1X PBS for 2x5 min.

Fluorescence in situ *hybridisation*

FISH was performed as described above (see section above: *Denaturation steps and hybridisation*).

5.3 Antibody design and production

Four species-specific antibodies were designed and produced to label the α– and β variants of the CENH3 proteins in hexaploid wheat and diploid barley. Our approach was to identify the most variable surface regions of the appropriate proteins followed by using the multiple sequence alignment tool of Clustal Omega (Sievers *et al.*, 2011); https://www.ebi.ac.uk/Tools/msa/clustalo/). Sequence alignment was based on the following UniProtKB (https://www.uniprot.org/) entries: wheat αCENH3 – A-genome: I3NV45, B-genome: I3NV43, D-genome: I3NV44 (Yuan *et al.*, 2015), barley αCENH3: G1APU2 (Sanei *et al.*, 2011); wheat βCENH3 – A-genome: A0A3B5Y4B2, B-genome: A0A3B5Z1Q8, *Aegilops tauschii* βCENH3 D-genome: A0A0G3YL56 (Yuan *et al.*, 2015), barley βCENH3: G1APU3 (Sanei *et al.*, 2011). Peptides having multiple matches with similar properties were excluded by the EMBOSS Matcher tool (EMBL-EBI) to avoid potential unwanted antibody cross-linking. All four peptide sequences were selected from the variable N-terminal tail domain of the wheat and barley CENH3 sequences.

The identified peptide sequences were synthetised, then HPLC and mass spectrometry analyses were performed to ensure the quality of peptides. The purified peptides were coupled to BSA or KLH carrier proteins before antibody production. Polyclonal antibodies were produced by injecting the conjugated peptides into animals for immunisation by a 90-day protocol (DC BioScience, Dundee, UK) and dribblets were collected for affinity purifications of the antibodies. The antibodies were raised against the following peptides:

1. NH_2-KKQLGPRPAQR-COOH, wheat αCENH3 in guinea pig (Wα)
2. NH_2-KRLRFELSPRWRP-COOH, wheat βCENH3 in rat (Wβ)
3. NH_2-KKIGSASSPSA-COOH, barley αCENH3 in sheep (Bα)
4. NH_2-CSKSEPQSQPKKKE-COOH, barley βCENH3 in rabbit (Bβ)

An anti-CENH3 antibody (against the CARTKHPAVRKTK peptide) universal for several species in the *Poaceae* (Li *et al*., 2013) was used as positive control.

Sample fixation, nuclei preparation and immunolabelling for the verification of the specificity of anti-CENH3 antibodies were performed as described in the respective points of the immunolabelling procedure (see above).

5.4 Confocal laser scanning microscopy

Detection of fluorescent signals was performed by an SP8 TCS confocal laser scanning microscope (Leica Microsystems). The DAPI-stained chromatin was detected between 410 and 470 nm after excitation at 405 nm. The settings for the applied fluorescent pigments conjugated to various secondary antibodies were as follows: Abberior StarGreen and Alexa Fluor 488 – excited at 488 nm, detected from 490 to 560 nm; Abberior StarOrange and Alexa Fluor 594 – excited at 561 nm, detected from 600 to 660 nm; Star Red and Alexa Fluor 647 – excited at 633 nm, detected from 650 to 700 nm. A series of confocal images ("*z* stacks") with a lateral (*x* and *y*) resolution of 45 nm and an axial (*z*) resolution of 200 nm were acquired by a HC PL APO CS2 63×/1.40 oil immersion objective (Leica). The size of confocal aperture was set to 1.35 Airy Unit (128.9 μm). Image acquisition was carried out by bidirectional scanning along the *x*-axis. Image stacks were subjected to deconvolution by Huygens Essential v18.04 (Scientific Volume Imaging). 3D reconstructions were obtained using the Leica Application Suite Advanced Fluorescence software v3.1.5.1638 or the Imaris multidimensional microscopy data analysis software v9.6 (Oxford Instruments).

6 RESULTS

6.1 Optimalisation of standard methodology for plant cell nuclei preparation

These limitations were addressed in the course of the present work by developing a simple nuclei preparation procedure where fixation was adjusted to preserve the 3D ultrastructure of the nucleus, while the cell wall/cytoplasm were removed mechanically, without the use of dedicated enzymes. To allow the easy transferability of the present procedure to other members of the research community including undergraduate or postgraduate students, manual maceration of the intact tissues was substituted with tissue grinding. This procedure was tested on interphase nuclei from root tips and male meiocytes of small grain cereals (wheat, barley, and rye) and specimens suitable for both immunolabelling and in situ hybridisation methods were obtained as well as for the combinations of these methods. The following measures were taken during the optimalisation of the nuclei preparation techniques:

6.1.1 Optimalisation of cell fixation

To efficiently remove the cell walls and the cytoplasm by mechanical force alone, fixation times (1 h in 4% PFA) used in previous standard procedure had to be shortened.

Shortening of the fixation time required an accelerated access of the fixative to the internal cell layers of the intact tissue. The effect of a detergent added to the non-denaturing fixative was thus tested to reveal whether it facilitates quick penetration of the fixative. Wheat and barley root tips were fixed for approx. 15 min in 4% PFA with

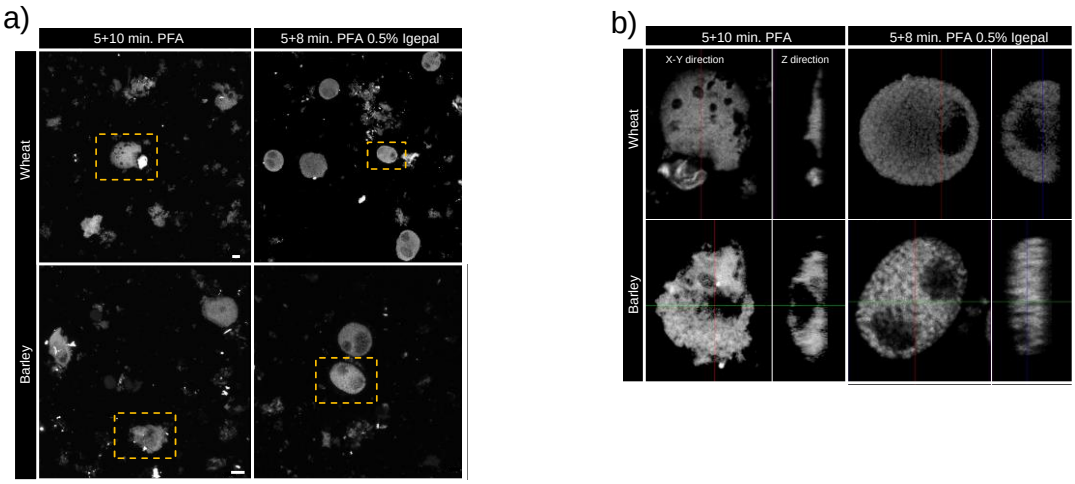
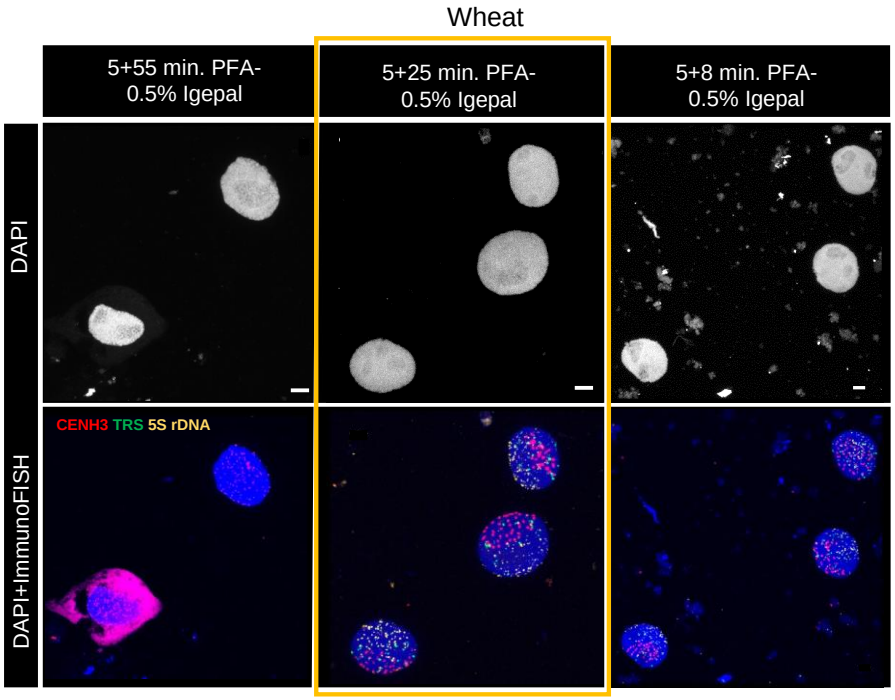


Figure 5. ***The effect of a non-ionic detergent (Igepal) included into the non-denaturing fixative (4% PFA) during the short fixation of wheat (top panels) and barley (bottom panels) root tips.***

(A) Left column: damaged somatic nuclei fixed without a detergent; right column: considerably improved nuclear morphology when fixed with the inclusion of 0.5% Igepal. (B) Enlarged and rotated images of the four nuclei highlighted by frames in Figure 5a. The x-y *and the* z *directions (left and right panel, resp.) show significantly improved 2D and 3D morphology upon the inclusion of 0.5% Igepal in the fixative. Bars = 5 µm.*

or without adding 0.5% of the non-ionic, and non-denaturing detergent Igepal to the fixative solution. Penetration was further aided by 5 min of vacuum infiltration in each treatments. Short fixation without the addition of the detergent resulted in damaged cells and a poor nuclear morphology for both wheat and barley root tissue (Figure 5A, left panel). The inclusion of the detergent (0.5% Igepal) improved nuclear morphology and reduced the number of damaged cells, although did not diminish it entirely (Figure 5A, right panel). The 3D structure of the nuclei and the integrity of the chromatin has significantly improved as well (Figure 5B). These experiments confirmed that the addition of the detergent allowed a better tissue fixation and shortens the required fixation time when using a non-denaturing fixative.

The next goal during the technique optimalisation was to define the extent of the fixation time which may result in a maximal number of nuclei with normal nuclear morphology and reduces the proportion of damaged cells, at the same time allowing a



good nuclear permeability.

Figure 6. The effect of three fixation times on the cell integrity and permeability of wheat mitotic nuclei as shown by DAPI counterstaining and immunoFISH.

First row shows nuclear morphology by DAPI counterstaining. Second row: ImmunoFISH reveals the nuclear localisation of CENH3 protein (red, marking functional centromeres), the telomeric repeat sequences (green) and the 5S rDNA repeats (orange). The chromatin is counterstained with DAPI (blue). 5 + 55 min of fixation provided a poor nuclear morphology and reduced cell permeability,

apparent from the low signal intensity and high background staining. 5+25 min of fixation with 4% PFA (including 0.5% Igepal) resulted in a good nuclear morphology and a low number of debris, which proved to be optimal for the applied molecular cytogenetic techniques (highlighted by a yellow box). Fixation for 5+8 min preserved nuclear morphology, but a high proportion of damaged cell fragments made cell imaging and signal interpretation difficult. Bars = 5 μm.

The following duration times were thus tested on wheat and barley root tips (Figures 6-7): 60 (5 min vacuum+55) min, 30 (5+25) min, and 13 (5+8) min. The 60 min fixation (Figures 6-7, first columns) resulted in an excellent 3D structure, but the mechanical forces applied after the fixation were not effective in removing the cytoplasm. ImmunoFISH (Figures 6-7, first columns, bottom images) showed high background staining and low signal intensity both for the antibody and the FISH probes, which confirmed the compromised permeability of the nuclei.

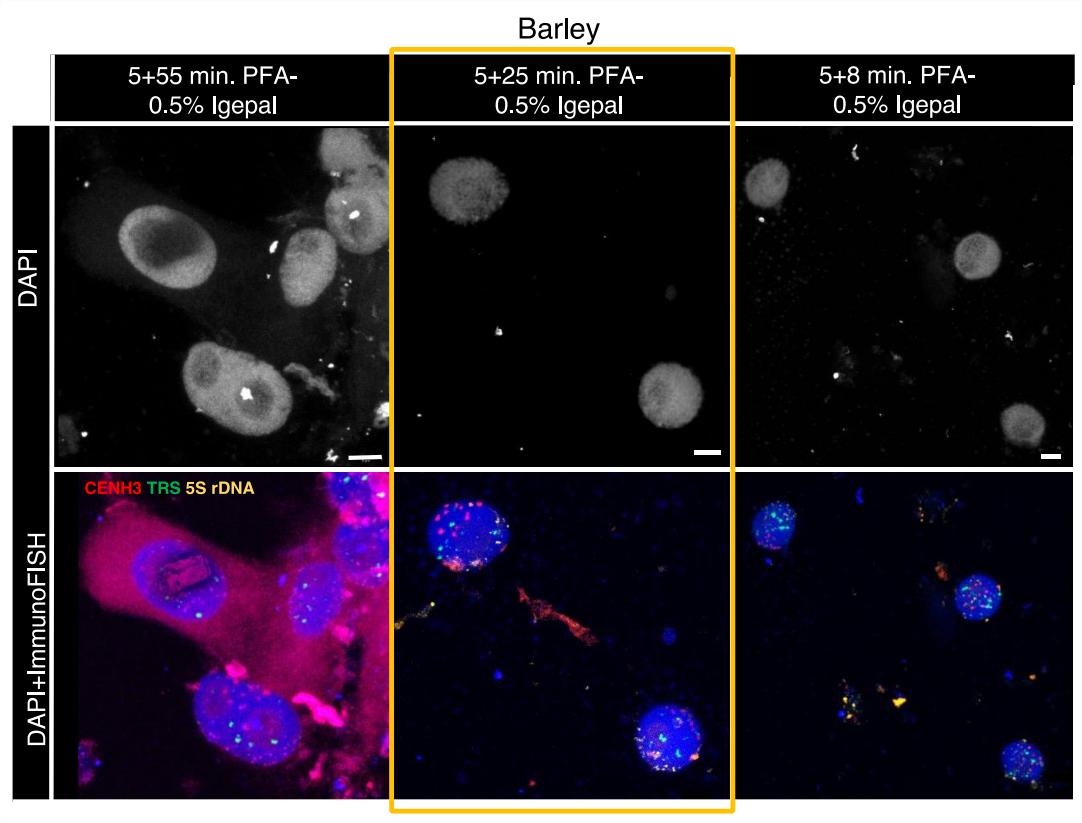


Figure 7. The effect of three fixation times on cell integrity and permeability of barley mitotic nuclei.

First row shows nuclear morphology by DAPI counterstaining. Second row: ImmunoFISH reveals the nuclear localisation of CENH3 protein (red), the telomeric repeat sequences (green) and the 5S rDNA repeats (orange). The chromatin is counterstained with DAPI (blue). 5 + 55 minutes of fixation with 4% PFA (including 0.5% Igepal) provided a poor nuclear morphology and reduced cell permeability, apparent from a large amount of cytoplasm around the nuclei, low signal intensity and high background staining. 5+25 minutes of fixation resulted in good nuclear morphology and a low proportion of debris, optimal for the applied molecular cytogenetic techniques (highlighted with a yellow box). Fixation for 5+8 minutes preserved nuclear morphology, but a high proportion of damaged cell fragments made cell imaging and signal interpretation difficult. Bars = 5 μm.

Fixations of 30 min and 13 min both preserved the nuclear structure and allowed a good antibody and probe penetration (Figures 6-7: second and third columns). The 30 min of fixation had the advantage of producing a lower proportion of damaged cells and debris, especially in wheat, and it was therefore considered as an optimal fixation time for the genotypes used in the present study.

6.1.2 Introducing mechanical tissue homogenisation

Tissue homogenisation in a dedicated grinding set (see the M&M section) allowed to produce large amounts of plant material in a short period of time without rigorous manual manipulations. Cells were, however, prone to form aggregates in suspensions, which prevented successful cytoplasm removal, effective cell filtration and caused high background staining in molecular cytology methods. To reduce cell aggregation, tissue grinding was performed in different solutions to identify the most suitable composition for cell separation.

Figure 8. The effect of three buffers applied during tissue grinding on the in-solution agregation of barley root tip cell nuclei. *Root tip cell nuclei were prone to form agregations when homogenised in PEM buffer or extraction buffer. Optimal separation of nuclei was obtained when homogenising root tips in LB01 buffer (results boxed in yellow). Bars = 5 μm.*

Three solutions, PEM (or BRB80) buffer (Borisy and Olmsted, 1972), extraction solution (0.2 M mannitol, 0.15 M glucose, 2 mM $CaCl_2$), and LB01 buffer (Doležel *et al*., 1989; see the M&M section) were tested for this purpose. Tissue grinding in the PEM buffer and the extraction solution resulted in large cell aggregates and hardly any individual nuclei in barley (Figure 8). Samples in LB01, however, contained a high proportion of individual nuclei indicating an adequate cell wall/cytoplasm elimination (Figure 8).

An overview of the optimised in solution nuclei preparation technique developed in the present work is presented in Figure 9. According to this root tips of wheat or barley

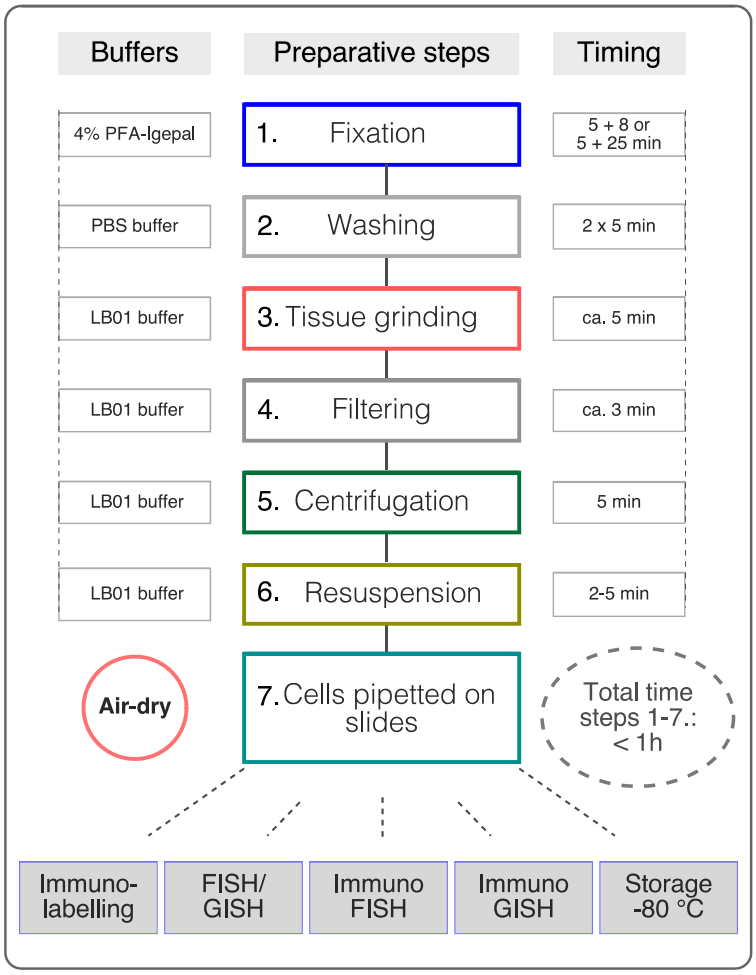


Figure 9. Flowchart of the nuclei preparation procedure suitable for protein visualisation by immunolabelling and localisation of nucleic acid sequences by in situ hybridisation.

The main steps of the procedure are numbered in the middle in coloured boxes. Buffers and the respective timing are indicated on the left and right side, respectively. Possible downstream applications are indicated in grey boxes in the bottom.

were fixed in 4% PFA for 30 min where the first five minutes involved vacuum infiltration (Figure 9, step 1). Root tips were subsequently washed in PBS buffer two times for 5 min (Figure 9, step 2) which was followed by tissue grinding in LB01 buffer (Figure 9, step 3). The homogenised cells were double-filtered through a 70-µm and a 40-µm strainer (Figure 9, step 4), then pelleted by centrifugation for 5 min at 2,000 × g (Figure 9, step 5). The pelleted nuclei were resuspended in LB01 buffer (Figure 9, step 6), pipetted on the surface of an adhesion slide and air dried (Figure 9, step 7). The slides were then directly processed for immunolabelling, *in situ* hybridisation or immunoFISH or stored at -80 °C up to several months.

6.2 (Sub)genome-specific antibody design and verification

6.2.1 Variant- and species-specific antibody design

Wheat and barley carry two CENH3 encoding genes, which produce the α– and βCENH3 histone variants. Polyclonal antibodies recognising the different CENH3 variants from wheat and barley were designed to analyse cross-species incorporation of the CENH3 protein variants in wheat × barley hybrids.

Percent identity matrixes of the wheat- and barley αCENH3 amino acid sequences revealed an identity of 77% while βCENH3 proteins shared a sequence identity of 69% between wheat and barley (Figure 10).

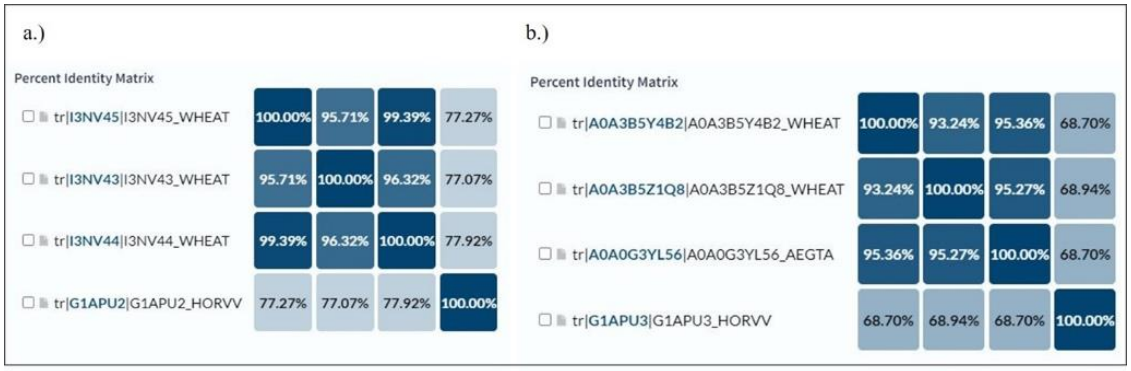


Figure 10. Percent identity matrixes of wheat and barley CENH3 protein variants α and β.

(A) *Amino acid sequence identity between wheat (UniProt accession numbers: A-genome: I3NV45, B-genome: I3NV43, D-genome: I3NV44) and barley (Uniprot accession number: G1APU2) αCENH3 protein variants.* ***(B)*** *Amino acid sequence identity between wheat (UniProt accession numbers: A-genome: A0A3B5Y4B2, B-genome: A0A3B5Z1Q8,* Aegilops tauschii *βCENH3 D-genome: A0A0G3YL56) and barley (Uniprot accession number: G1APU3) βCENH3 protein variants.*

Despite the high similarity score, multiple sequence alignments revealed a short polymorphic region within the N-terminal tail of the CENH3 protein (Figure 11).

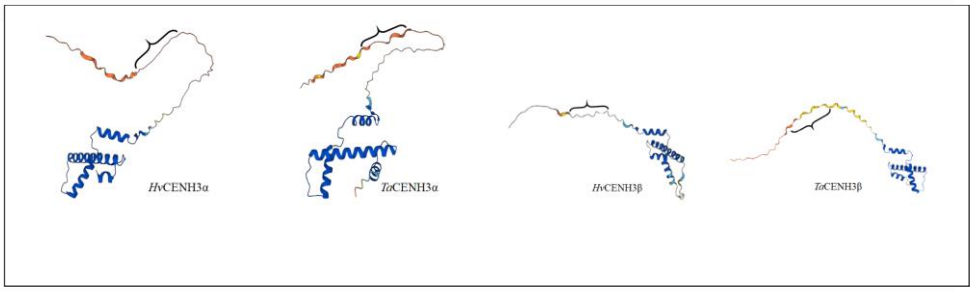


Figure 11. Predicted tertiary protein structure of the wheat and barley CENH3 variants by the AlphaFold Protein Structure Database.

The positions of the aminoacid sequences used for polyclonal antibody production are indicated for each protein variants with brackets.

Peptide sequences differing between the corresponding wheat and barley CENH3 variants (Figure 12) were subsequently selected for species- and variant-specific polyclonal antibody production (see Materials and Methods).

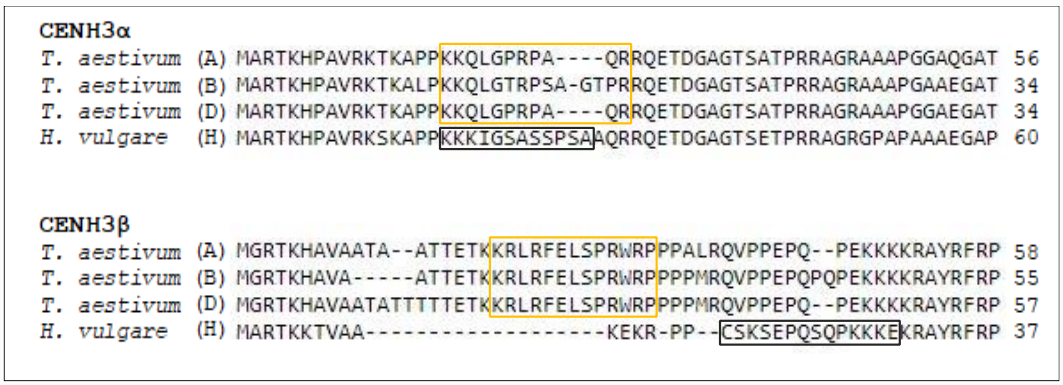


Figure 12. Multiple sequence alignment of the wheat and barley α– and βCENH3 proteins.

Sequences were compared by ClustalW (Lasergene). The position used for construction of the peptide antibodies against wheat α– and βCENH3 is boxed in yellow. The peptide sequence used for the barley α and βCENH3 antibody production is boxed in black.

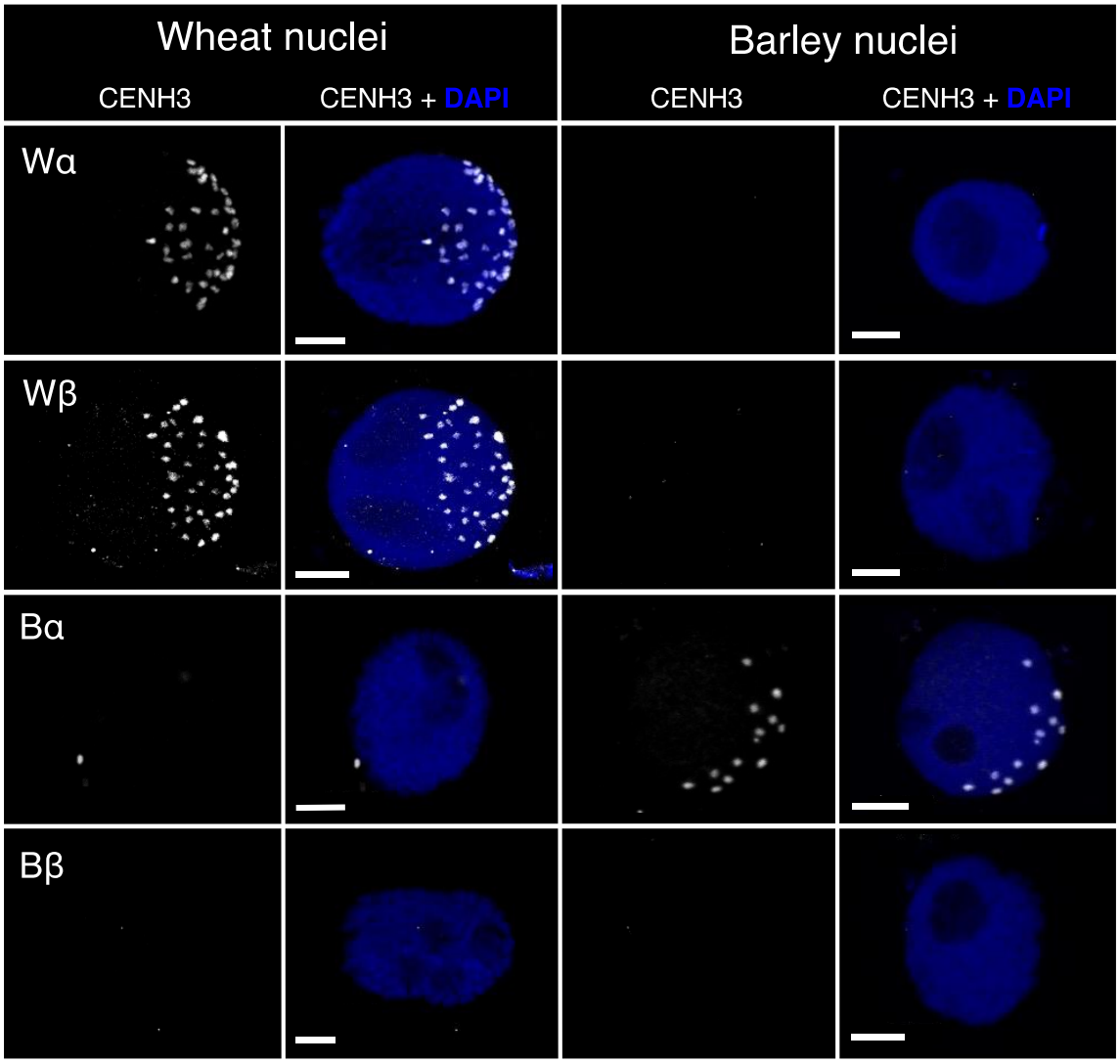
6.2.2 Validation of wheat and barley α– and βCENH3 antibodies

The obtained four polyclonal antibodies were subsequently tested for indirect immunofluorescence on wheat and barley somatic (root tip) nuclei. Immunolabelling with the anti-wheat alpha CENH3 (abbreviation: Wα) and anti-wheat beta CENH3 (Wβ) antibodies revealed 22-39 dot-like signals on wheat root-tip nuclei (n=29). No signal was observed on nuclei of barley when using the anti-wheat CENH3 antibodies, revealing the potential species-specificity of the antibodies. Immunofluorescence with the anti-barley alpha CENH3 (Bα) antibody produced 7-14 dot-like fluorescent signals on barley root tip nuclei, whilst no signal was detected on wheat nuclei. The fluorescence signal in each case was organised within one nuclear hemisphere, in the vicinity of the nuclear periphery (Figure 13), characteristic of centromere organisation within the nucleus. The anti-barley beta (Bβ) antibody showed a very faint or no specific fluorescent signal in either barley or wheat (Figure 13), indicating its inefficiency in recognising the epitope by the immunohystochemical methods used in this study. The anti-barley beta (Bβ) antibody was thus omitted from further cytological analysis.

To examine if the nuclear signal given by the CENH3 protein variant antibodies designed in this study corresponds to the core centromeres, we performed immunoFISH where CENH3 histone proteins and the centromeric DNA were labelled simultaneously. The centromeric retrotransposon of wheat (CRW), a long terminal

repeat (LTR) retrotransposon, specific to the core centromere was visualised together with either Wα or Wβ CENH3 histone variants.

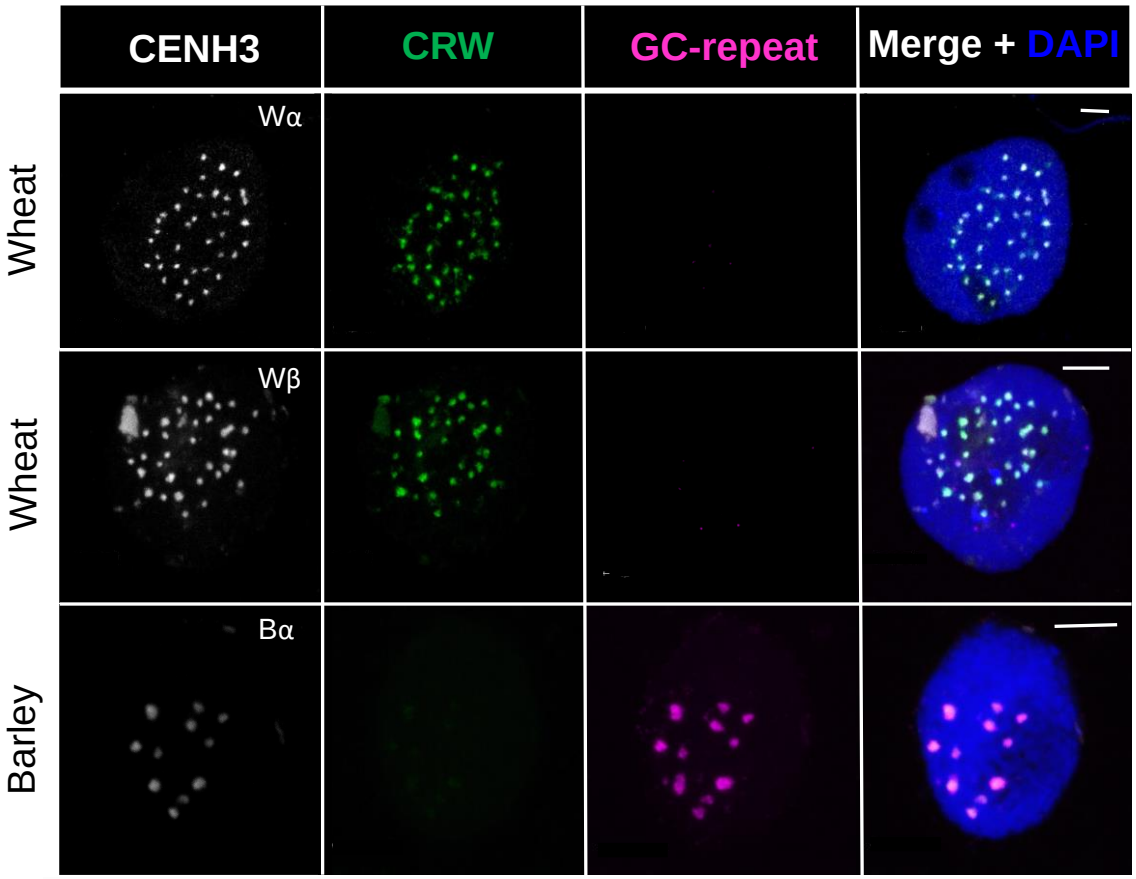
Figure 13. Immunolocalisation of CENH3 protein variants on wheat and barley root tip nuclei by using the four variant- and species-specific antibodies designed in the present work.



Rows represent experiments with wheat α (Wα), wheat β (Wβ), barley α (Bα) and barley β (Bβ) CENH3 antibodies applied to wheat and barley nuclei. Antibodies designed to recognise α and βCENH3 variants of wheat proved to be species-specific as they produced signal only on wheat nuclei (white signal on the left panels) and failed to give signal on barley nuclei (no signal on the right panels). The antibody aimed to recognise αCENH3 of barley proved to be species-specific as it failed to produce signal on wheat nuclei (no signal on the left panels) and white signal on the right panels). The antibody designed to detect the barley βCENH3 protein failed to produce signal on either wheat or barley nuclei, showing its inability to track barley βCENH3. Bars = 5 μm.

Signal produced by the Wα and Wβ CENH3 antibodies colocalised with the CRW retrotransposon on wheat somatic nuclei, indicating that the antibodies detected the centromeric region in wheat (Figure 14). To visualise DNA of the barley centromere the G+C microsatellite sequence was amplified by PCR and used as a FISH probe (Figure 14). Bα CENH3 fluorescence labelling colocalised with the FISH signal given

by the G+C repeats on barley somatic nuclei, indicating that the Bα CENH3 antibody recognises the centromeric region of barley chromosomes (Figure 14). These results confirmed that the Wα, Wβ and Bα CENH3 antibodies detect the centromeric regions



in the corresponding species and they are thus suitable for further examination of the parental centromere function in wheat × barley hybrids.

Figure 14. Centromeric localisation of CENH3-specific antibodies in wheat and barley interphase nuclei by co-localisation with centromere-specific FISH probes.

Centromeres were immunolabelled by wheat α (Wα), wheat β (Wβ) and barley α (Bα) CENH3-specific antibodies (white) while the centromeric retrotransposon of wheat (CRW, green) and the barley centromere-specific G+C-rich satellite (magenta) were detected by FISH. The chromatin was counterstained with DAPI (blue on merge). Wheat α and βCENH3 protein signals colocalise with the FISH signal given by the CRW probe indicating that the antibodies designed in the present study show centromere-specific signals. Barley αCENH3 colocalises with the barley centromere-specific G+C-repeat, indicating the centromere-specificity of the barley αCENH3 antibody Bars = 5 µm.

6.3 Production and cytological characterisation of wheat × barley F1 hybrids

6.3.1 Development of wheat × barley F1 hybrids

To explore the capacity of centromeric DNA to load CENH3 originating from a related species (cross loading) in interspecific hybrids we developed wheat × barley F1 hybrids where somatic cells carried both wheat and barley chromosomes in different

combinations. Wheat × barley F1 hybrids were produced by crossing the doubled haploid 'M1' wheat with the spring barley variety 'Golden Promise' (see Materials and Methods). 'M1' wheat florets (n=960) were emasculated and outcrossed with barley according to the method described by Polgári *et al*. (2014). Eighteen embryos were rescued at the 14th day after pollination, of which 16 plants regenerated on N6D medium (Chu *et al*., 1975). Cytological examinations were carried out on root tips collected from potted plants.

6.3.2 Cytological characterisation of two wheat × barley hybrids

In barley, the α– and βCENH3 variant encoding genes are located on the chromosomes 1H and 6H, respectively (Sanei *et al.*, 2011), while in wheat both genes are located on the chromosomes of the homoeologous group 1 (Yuan *et al.*, 2015). Two of the obtained sixteen F1 hybrid plants were selected for further cytological analyses based on cytological techniques showing the presence and absence of the barley chromosomes carrying the CENH3-encoding sequences.

Cytological examination of root tip cells of the partial hybrid No. 22/2020 (n=75) by simultaneous GISH-FISH using barley genomic DNA and the 5S rDNA as probes revealed 21 wheat chromosomes and confirmed the presence of four barley chromosomes in the somatic cells of the F1 hybrid.

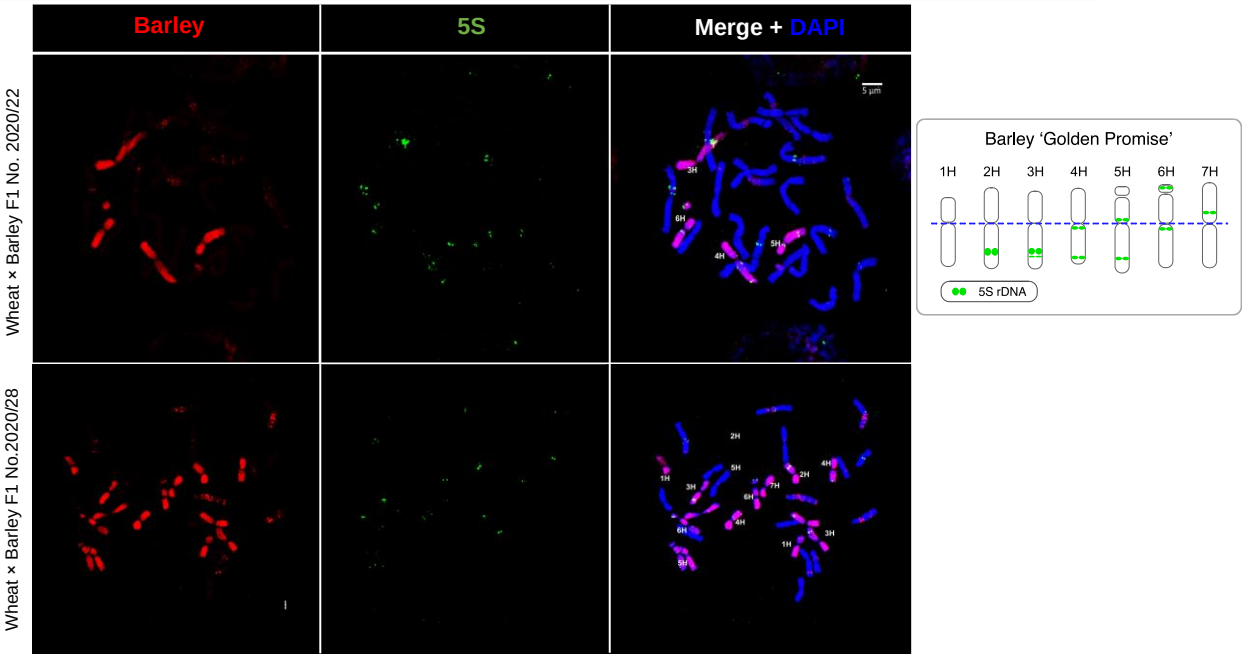


Figure 15. Molecular cytological characterisation of the two primary (F1) wheat × barley hybrids studied.

Left panels: The barley genome was visualised by GISH (labelled in red). Individual barley chromosomes were identified by FISH using a 5S rDNA-specific probe (labelled in green). Barley chromosomes were identified according to the karyogramme presented on the right panel. The

chromatin was counterstained with DAPI (blue on merge). Bar = 5 µm. Right panel: Schematic karyogramme of barley 'Golden Promise' according to the 5S rDNA-specific FISH signal distribution.

The 5S probe identified the barley chromosomes as 3H, 4H, 5H and 6H (Figure 15), confirming the absence of the 1H chromosome, and thus the absence of the barley αCENH3 encoding gene.

In situ hybridisation of the No. 28/2020 hybrid detected 14 barley chromosomes (Figure 15), revealing the duplication of the paternal genome (n=48). A varying number of wheat chromosomes were detected beside the barley chromosomes. In the majority of cells analysed (79%) the number of wheat chromosomes ranged from 14 to 20, indicating mitotic instability and elimination of the wheat chromosomes. In a subset (21%) of mitotic nuclei analysed only the chromosomes originating from the barley parent were retained while the wheat chromosomes were fully eliminated (Figure 16).

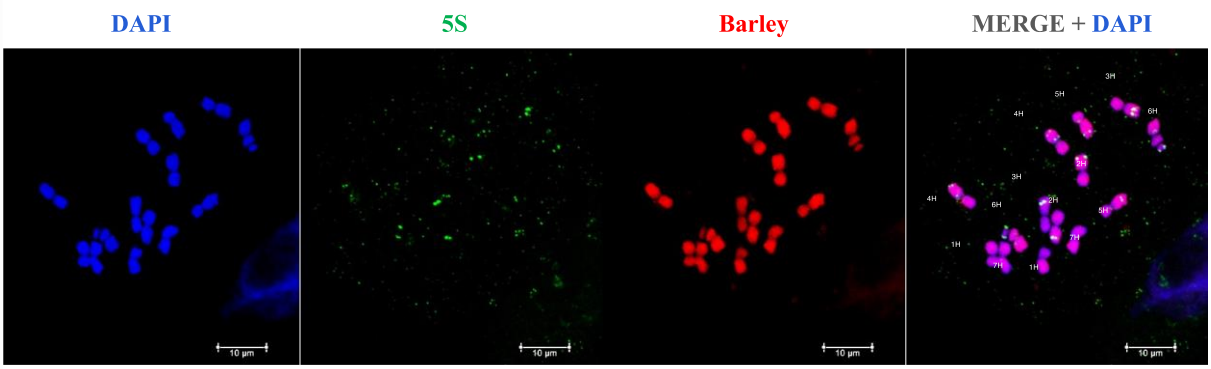


Figure 16. Combined genomic* in situ *hybridisation (GISH) and fluorescence* in situ *hybridisation (FISH) on a metaphase chromosome spread prepared from root tip cells of the wheat ×barley hybrid No. 28/2020.

FISH with the 5S rDNA probe (green) and GISH with total genomic DNA of barley as probe (red) reveals the presence of the full barley chromosome set and the complete elimination of the wheat chromosome sets. Bars = 10 µm.

6.4 Centromere organisation in mitotic cells of wheat × barley F1 hybrids

6.4.1 CENH3 loading in the mitotic cells of the F1 hybrid No. 22/2020

The loading of wheat and barley CENH3 proteins into the parental centromeres was evaluated in the partial F1 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 (Figure 15). 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 endogeneous 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 11-24 wheat centromeric signals and 2-4 barley centromeric signals (n=44, Figure 17). 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 (Figure 17, yellow arrows) revealing association between the centromeres of the two parental species.

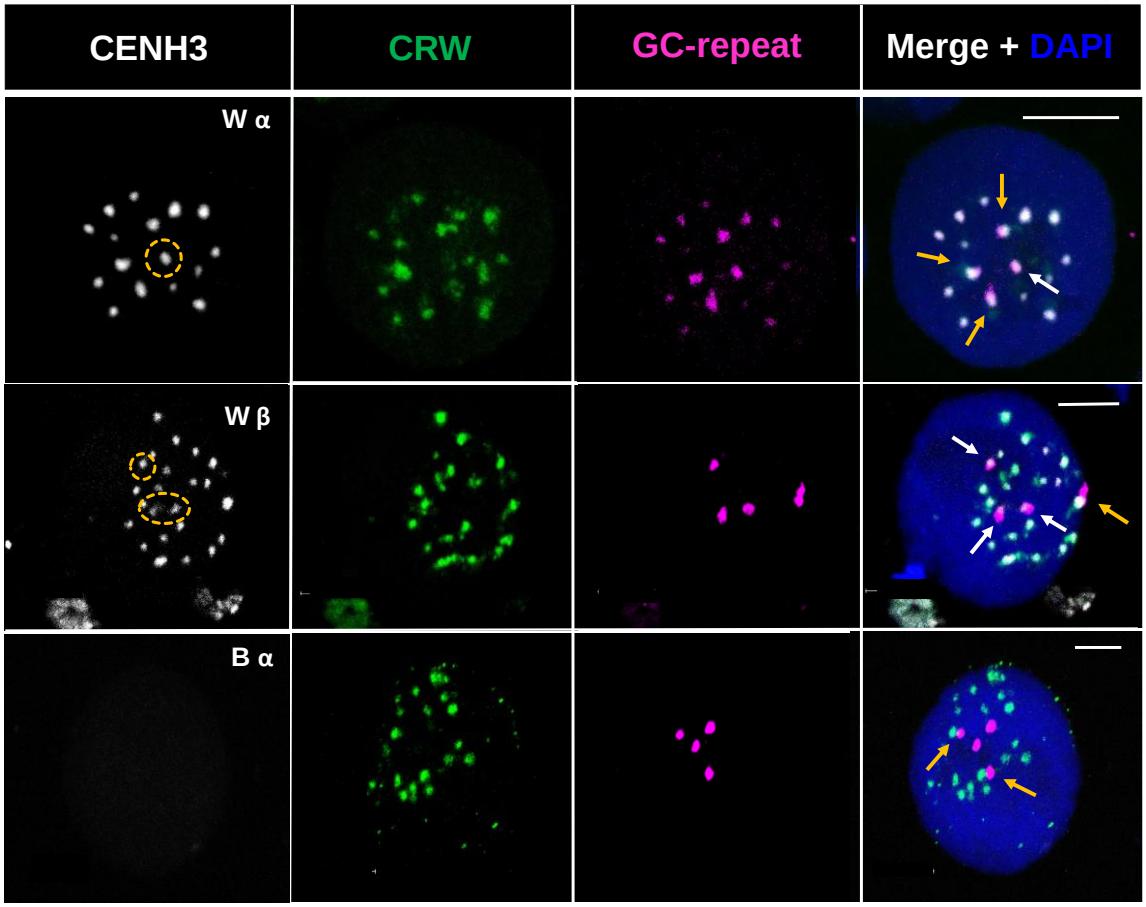


Figure 17. Immunolocalisation of CENH3-variants in mitotic nuclei of wheat × barley hybrid No. 22/2020. *Centromeres were immunolabelled by CENH3-specific antibodies (white) while the centromeric retrotransposon of wheat (CRW, green) and the barley centromere-specific G+C-rich satellite (magenta) were detected by FISH. The chromatin was counterstained with DAPI (blue on merge). Yellow arrows indicate colocalisation of wheat and barley centromeres. White arrows highlight barley centromeres located individually, also encircled as loading wheat CENH3 proteins on the left panel. Bars = 5 µm.*

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 (encircled centromeres on Figure 17, left panels) or in association with the wheat centromeres (Figure 17, yellow and white arrows). 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 (Figure 17), revealing that barley centromeric repeats are capable to load CENH3 proteins from a related species.

6.4.2 CENH3 loading in the mitotic cells of the wheat × barley F1 hybrid No. 28/2020

Cytological analysis of the F1 hybrid No. 28/2020 showed 14 barley chromosomes, representing the full diploid genome of barley (1H-7H, Figure 15) beside a variable number (0-20) of wheat chromosomes (Figures 15-16). 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, Figure 18).

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 (Figure 18). In contrast, barley centromeres showed a clear Wα CENH3 signal, indicating that Wα CENH3 is transcribed in the hybrid nuclei.

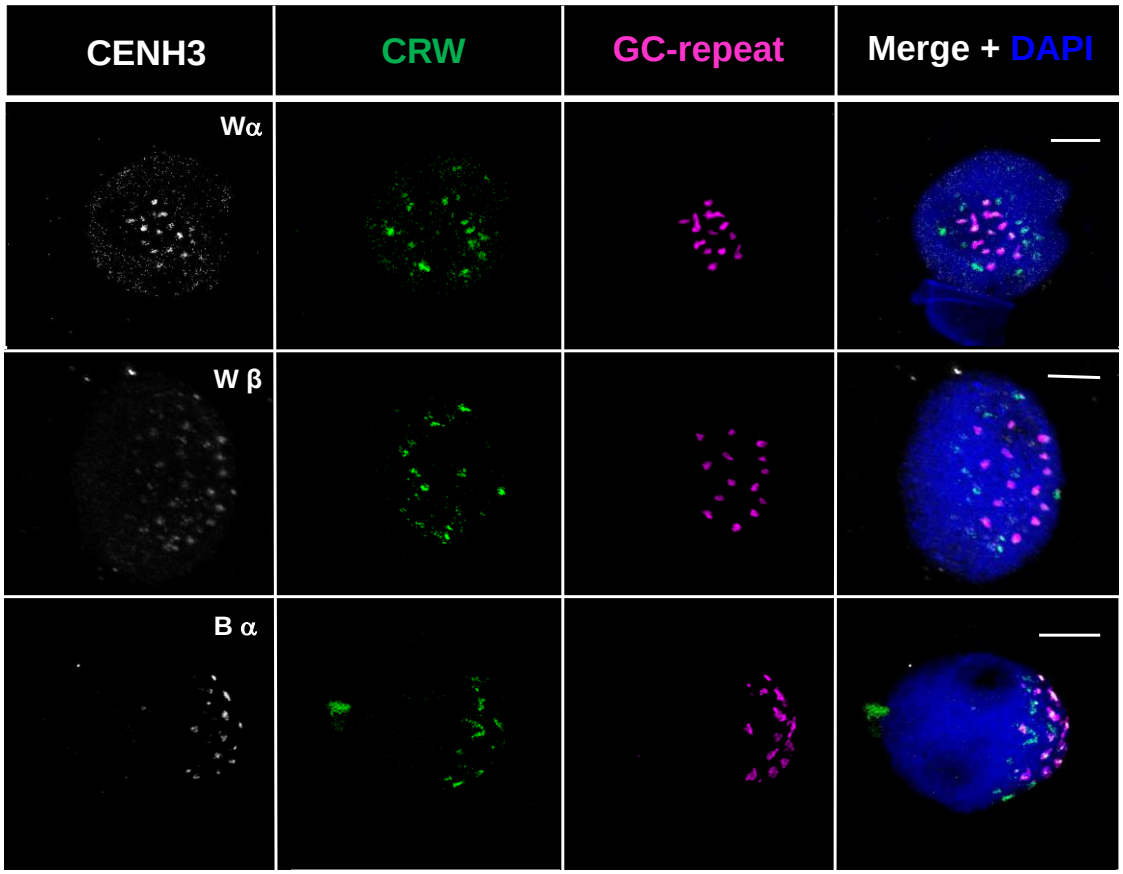


Figure 18. Immunolocalisation of CENH3-variants in mitotic nuclei of wheat × barley hybrid No. 28/2020. *Centromeres were immunolabelled by CENH3-specific antibodies (white) while the centromeric retrotransposon of wheat (CRW, green) and the barley centromere-specific G+C-rich satellite (magenta) were detected by FISH. The chromatin was counterstained with DAPI (blue on merge). Bars = 5 µm.*

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 (Figure 18).

6.5 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 (Figure 19).

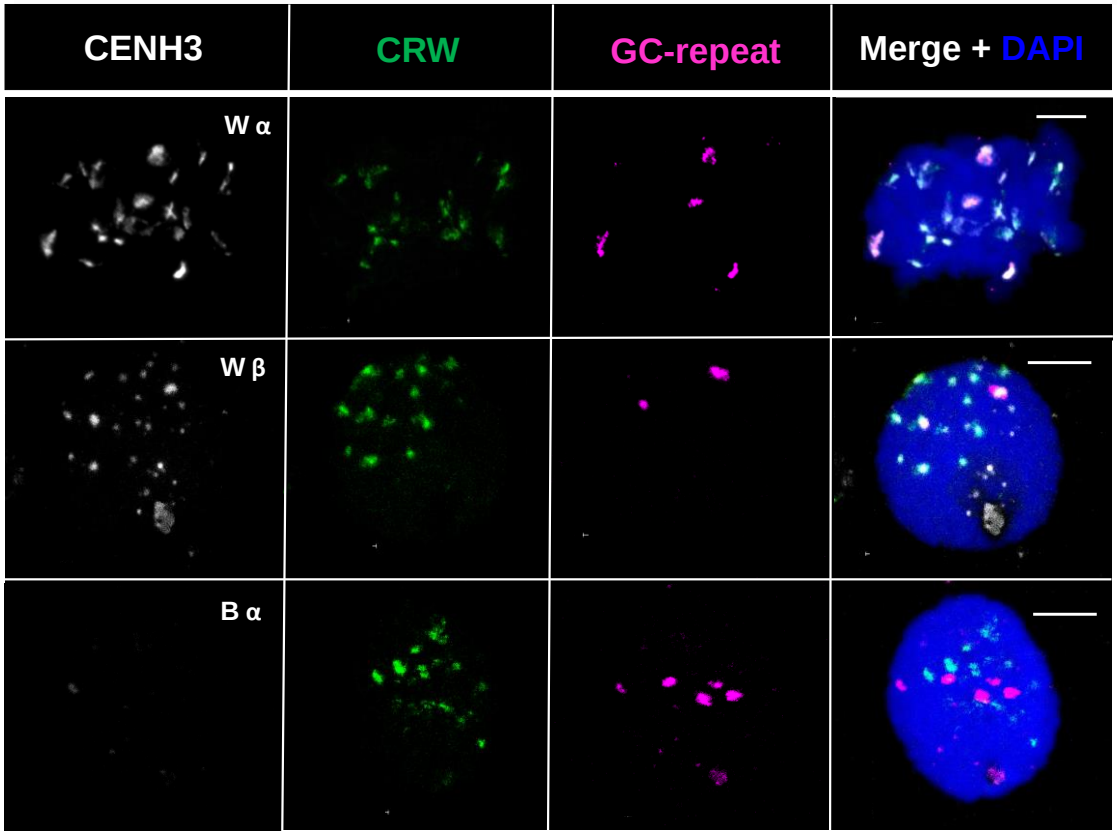
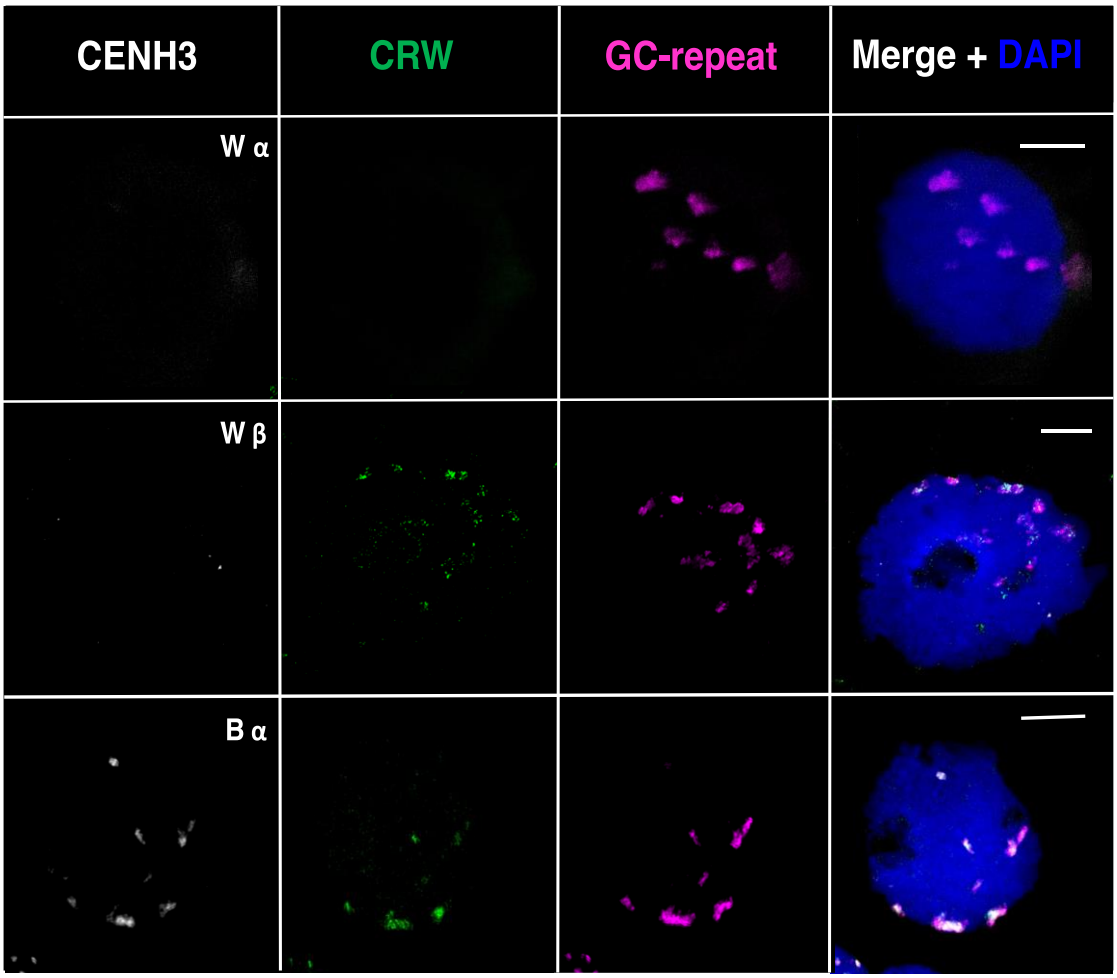


Figure 19. Immunolocalisation of CENH3-variants in meiotic nuclei of wheat × barley hybrid No. 22/2020. *Centromeres were immunolabelled by CENH3-specific antibodies (white) while the centromeric retrotransposon of wheat (CRW, green) and the barley centromere-specific G+C-rich satellite (magenta) were detected by FISH. The chromatin was counterstained with DAPI (blue on merge). Bars = 5 µm.*

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

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.

Figure 20. Immunolocalisation of CENH3-variants in meiotic nuclei of wheat × barley hybrid No. 28/2020. *Centromeres were immunolabelled by CENH3-specific antibodies (white) while the centromeric retrotransposon of wheat (CRW, green) and the barley centromere-specific G+C-rich satellite (magenta) were detected by FISH. The chromatin was counterstained with DAPI (blue on merge). Bars = 5 µm.*



7 DISCUSSION

7.1 Optimalisation of standard methodology for plant cell nuclei preparation

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 optimalisation 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 optimised before the procedure could be applied consistently.

Formaldehyde is the oldest fixative (Blum 1893), and, despite its toxicity and carcinogenicity, it is still one of the most popular fixatives and reported to outperform its proprietary alternatives (Boon and Kok, 2008). During the present work, the typical PFA fixation times of several hours used in traditional protocols (O'Brien *et al.*, 1973; Motte *et al.*, 1988) and 1 h in our standard procedure (Sepsi *et al.*, 2018) were reduced to 15-30 min so that the immunoreactivity of nuclear protein epitopes could be better preserved. The short fixation times imply only covalent bonding of PFA instead of its usual cross-linking effect between proteins and nucleic acids (Thavarajah *et al.*, 2012; Rhodes, 2013), which may significantly compromise immunodetection. To compensate for the shortened fixation time and enhance the penetration of PFA a detergent was added to the fixative solution. As an added benefit, this modification keeps the chromatin in a relaxed state (Vázquez-Nin *et al.*, 1992; Echeverría *et al.*, 1999), thus counteracting its artificial condensation during the treatments of the cytological procedures. Previously, the non-ionic and non-denaturing detergent Nonidet P-40 was occasionally added to fixatives (Kobayashi *et al.*, 1999). Since Nonidet P-40 has been no more available, the equivalent Igepal CA-630 (Sinha *et al.*, 2017) was applied here.

Due to failed attepts to standardise enzymatic treatments historically used to break down the plant cell walls; a mechanical removal of cell walls was experimented in the present research. PFA preserves proteins around the DNA fibres making them less accessible to labelled FISH/GISH probes. Additionally, the interphase/prophase chromatin is enclosed in the double membrane of the nuclear envelope, which is well preserved by the non-denaturing fixative and thus act as a barrier to the labelled probes

and protein antibodies. Mechanical disintegration of plant tissues for nuclei extraction has long been elaborated and adapted to immunolabelling as well as *in situ* hybridisation (Pendle and Shaw, 2016). In our hands, the tissue grinder originally developed for soft tissues (Dounce *et al.*, 1955) delivered reproducible results when combined with subsequent filtering to remove cell debris. This debris is considered to cause non-specific antibody/probe binding and thus, as a source of background/noise signal, hampers the generation of high-resolution images.

The simple in-solution approach developed here, required the utilisation of a buffer supporting nuclear integrity. Out of three solutions commonly used for nuclei handling, the LB01 buffer appeared outstanding in this respect. The low aggregation of nuclei and reproducible signal intensities observed with LB01 can be attributed to its components (see the M&M section). The monovalent cations Na+ and K+ are known to stabilise chromatin structure (Barros *et al.*, 2009) by preventing its intense condensation (Stellwagen *et al.*, 2011). The polyamine spermine can substitute divalent cations in stabilizing nuclear proteins, while the chelator EDTA inhibits phenol oxidases and nucleases by immobilizing their metal cofactors (Mg^{2+}, Mn^{2+}, and Cu^{2+}). The inhibition of these enzymes is important in protecting nuclear proteins and the DNA from oxidation and denaturation. The non-ionic surfactant Triton X-100 aids the lysis and solubilisation of plastid membranes (Guilfoyle, 1995) and thus contributes to decreased cytoplasmic contamination.

Compared with the alternatives currently available the present procedure is simple, easily transferable, and feasible for non-experts in the field of cytology. The omission of the cell wall degradation step and of the manual preparation makes the procedure more reliable and time efficient. The total duration of the procedure is about half as much (ca. 45 min vs. 90 min) with a similar hands-on time as in our standard procedure (Sepsi *et al*., 2018).

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.

7.2 Incorporation of parental CENH3 proteins into the centromeres in somatic cells of wheat × barley F1 hybrids

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. The *cereba* retroelement is highly conserved within the grass centromeres (Qi *et al.*, 2013; Presting *et al.*, 1998). Individual copies of the barley *cereba* and the wheat *cereba* orthologue, CRW share a sequence homology of 85% (Liu *et al.*, 2008). The *in situ* hybridisation probe designed to localise wheat centromeric DNA in the wheat × barley hybrid nuclei targeted the integrase region of the CRW sequence and was highly effective in detecting wheat centromeres whilst producing no (mitotic cells) or only minor (meiotic cells) signals on the barley centromeres. Centromeric DNA of barley was visualised by using the highly abundant, barley-specific G+C-rich satellite probe.

Cytological characterisation showed that the F1 hybrid 22/2020 carried the haploid chromosome set of wheat and chromosomes 3H, 4H, 5H and 6H of barley, lacking the chromosome (1H) encoding the alpha variant of barley CENH3 protein. 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 (here barley αCENH3) as outlined below.

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). Furthermore, although

CENH3 proteins are divergent even between closely related species it has been shown that they can functionally complement one another between distant phylogenetic groups. For instance, a CENH3 null mutant *Arabidopsis thaliana* is able to load orthologous CENH3 variants from progressively distant species to complement for the lack of the endogenous CENH3 protein, thus restoring plant growth and fertility (Maheshwari *et al*., 2015).

The gradual elimination of the wheat chromosomes was evident from the cytological examination of the somatic nuclei of the hybrid No. 28/2020. Unexpectedly, the full paternal chromosome set ($2n=2x=14$) was detected in somatic metaphase preparations, which may be a result of male meiotic restitution (De Storme and Geelen, 2013). Wheat chromosome elimination coincided with a specific nuclear localisation, where wheat centromeres surrounded the barley centromeres that were organised into one group at the nuclear periphery. While wheat αCENH3 was present on barley centromeres, only a poor intensity staining could be observed on the exteriorised wheat chromosomes. This was in agreement with data on *H. vulgare* × *H. bulbosum* hybrids where paternal chromosome elimination coincided with a peripheral centromere compartmentalisation and the loss of CENH3 (Sanei *et al*., 2011). This observation is widely supported with the known peripheral pattern of spatial localisation of the parental chromosome set destined to be eliminated in mitoses (Leitch *et al.*, 1991; Schwarzacher *et al.*, 1992; Mochida *et al.*, 2004; Kim *et al.*, 2002) and even the interphase (Gernand *et al.*, 2005) in various interspecific cereal hybrids. CENH3 unloading and nuclear chromosome exteriorisation may thus be a conserved strategy for genome elimination within the *Triticeae* tribe. In the somatic nuclei of the hybrid No. 28/2020 wheat βCENH3 was incorporated into the wheat and barley centromeres. Barley αCENH3 was incorporated into the barley centromeres but could not be detected in the wheat centromeric DNA. The lack of barley αCENH3 from the wheat centromeres may be resulted by a structural inability of wheat centromeres to incorporate barley CENH3 proteins or by a progressive epigenetic chromosome silencing leading to CENH3 unloading. The capacity of the stable barley centromeres to incorporate both wheat and barley CENH3 provides important evidence that CENH3 proteins from the two parental species can be co-loaded into the same centromeric DNA and still maintain chromosome stability at mitosis. This is in contrast with data in *Arabidopsis* where CENH3 from a species as distant as *Zea mays* can functionally complement the CENH3 null mutant, however when complemented plants were crossed to the wild type, naturally evolved divergence in the hybrid

progeny caused extensive mis-segregation, aneuploidy and fertility losses (Maheshwari *et al.*, 2015). Mis-segregation involved chromosomes with divergent CENH3s, which suggested that the essential function of CENH3 is conserved across distant species, but co-loading of diverse CENH3s weakens centromeres, leading to genome elimination. Our study showed that wheat and barley CENH3 proteins are incorporated within the same centromeric regions (by loading both wheat CENH3 and at least barley αCENH3). Co-loading of wheat and barley CENH3 variants did not affect the maintenance of the duplicated barley genome, but coexistence of the diverse CENH3 proteins within the same nucleus coincided with the apparent instability of the wheat genome.

7.3 Incorporation of parental CENH3 proteins into the centromeres in meiotic cells of wheat × barley F1 hybrids

Analysis of the meiotic cells of the hybrid No. 22/2020 led to the detection of a highly similar CENH3 loading pattern as observed in the mitotic cells. Two-four barley centromere-specific signal indicated that four barley centromeres are still present in meiotic prophase I of the hybrid. The varying number of wheat centromeric signals observed on meiotic prophase nuclei was in agreement with the wheat centromeres to undergo centromere-centromere associations during meiotic prophase I (Sepsi *et al.*, 2017).

By the onset of meiosis within the anthers of hybrid No. 28/2020 the wheat chromosome set became eliminated as demonstrated by the complete lack of wheat α– and βCENH3 signals. In contrast, the barley αCENH3 gave high numbers of strong immunosignals in the barley centromeres suggesting the elevated expression of barley *CENH3* genes and/or their higher copy number due to the presence of the full diploid genome as demonstrated. Together with the flexibility of barley centromeric DNA to load both the conspecific and wheat CENH3 this elevated CENH3 level may render barley centromeres dominant over those of wheat in the hybrid No. 28/2020.

7.4 Patterns of CENH3 incorporation in interspecific cereal hybrids

The potential scenarios for (cross-)loading of CENH3 proteins in the centromeric DNA of interspecific hybrids can be arranged as follows (Fig. 21): mutually exclusive, i.e., preserving the parental loading pattern (No. 1 in the figure), and mutually inclusive in both parents (permitting cross-specific CENH3 incorporation, No. 2) or unilaterally inclusive, i.e., for either of the two parents (Nos. 3-4). These scenarios may be specific

for each of the CENH3 variants and therefore should be evaluated separately in the presence or absence of their counterpart variant(s) in both somatic and meiotic nuclei.

Our data point to the existence of scenario No. 3 in the interspecific combination of wheat × barley, i.e., the unilateral preference for the two maternal, wheat CENH3 variants in the absence of barley αCENH3 as well as in the presence of the two barley CENH3 variants, both in somatic and (possibly) meiotic nuclei.

These scenarios can also be compared to published experimental data on other cereal cross combinations. Based on immunolabelling as direct evidence for CENH3 incorporation, the data can be distributed in three groups according to the type of material used: primary hybrids, established aneuploid (chromosome additions) or euploid (substitutions, translocations) genetic stocks, and transgenically produced alien CENH3 combinations.

In the two interspecific hybrid combinations tested so far, *Hordeum vulgare* × *H. bulbosum* (Sanei *et al*., 2011) and oat × pearl millet (Ishii *et al.*, 2015a), the maternal CENH3 was incorporated into all the paternal centromeres in somatic nuclei. In barley, both *H. vulgare* CENH3 variants effectively occupied the *H. bulbosum* centromeres in the presence as well as in the absence of the conspecific CENH3 proteins.

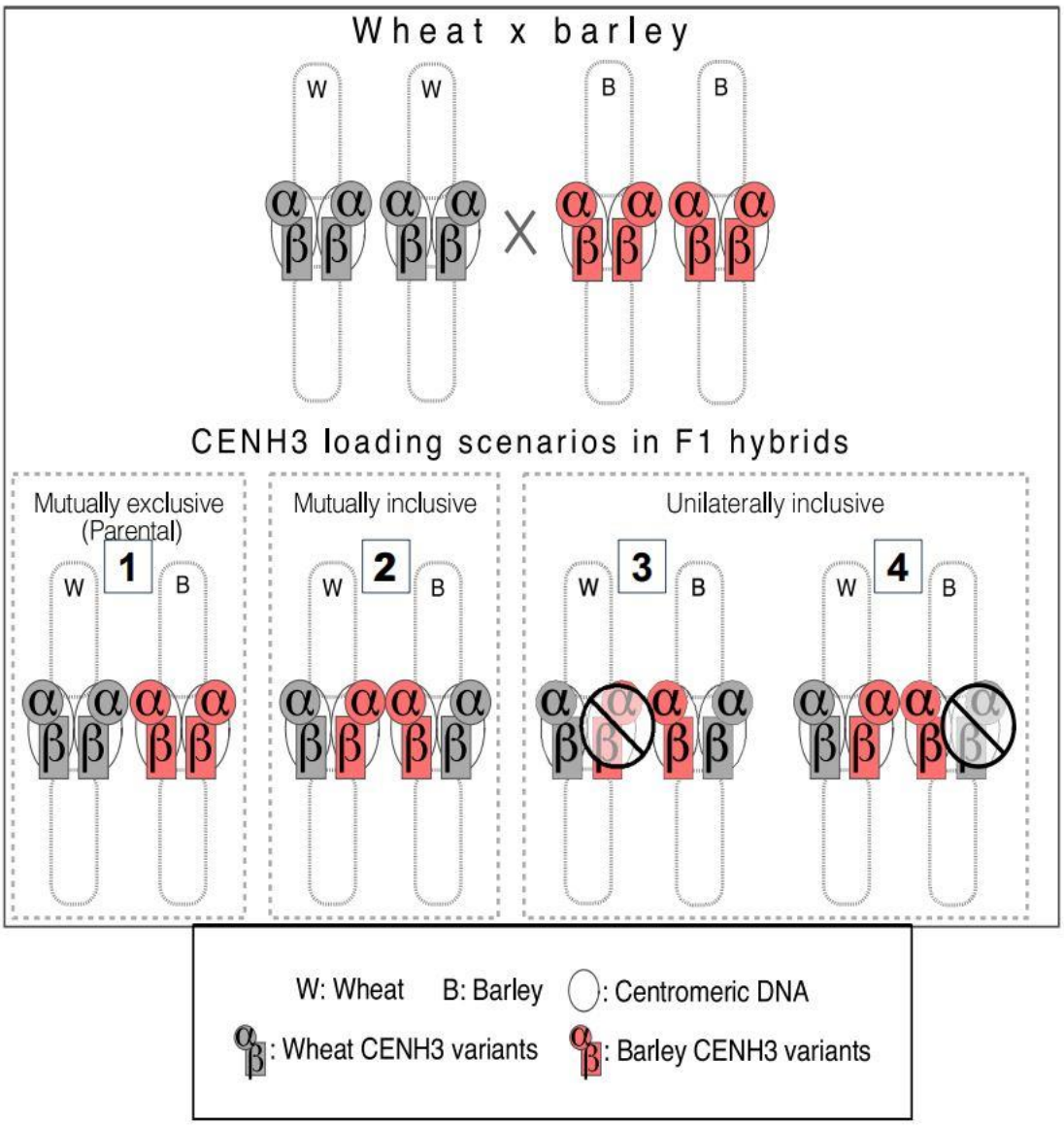


Figure 21. Schematic presentation of potential scenarios for CENH3 incorporation into the centromeres of wheat × barley F1 hybrids.

Stable genetic stocks can be viewed as end products mirroring the prior elimination process. Four derivative types from interspecific crosses have so far been studied by CENH3 immunolabelling of somatic nuclei, metaphase chromosomes, and chromatin fibres: (i) a disomic 7H chromosome substitution of *H. bulbosum* in barley (Sanei *et al.*, 2011), (ii) disomic additions of maize chromosome 3 (Wang *et al.*, 2014) and chromosome 6 (Jin et al. 2004) to oat, (iii) disomic substitutions of *Thinopyrum elongatum* (Guo *et al.*, 2016) and *Th. intermedium* (Li et al. 2023) chromosomes in wheat. Finally (iv), several wheat-rye lines were also tested: monosomic 2R and 6R chromosome addition lines (Guo et *al.*, 2016), over 100 1RS.1BL translocation lines containing hybrid wheat-rye centromeres (Wang *et al.*, 2017), and similar hybrid

centromeres in a reconstructed wheat 1B chromosome (Karimi-Ashtiyani *et al.*, 2021). In all these cases, a clear maternal (barley, oat, and wheat) CENH3 incorporation was observed into the remaining individual paternal chromosome centromeres either in the presence (Jin et al. 2004) or the absence of their own *CENH3* genes. The only exception to this scenario was the double-disomic 1H+6H barley chromosome addition line to wheat: here all four *CENH3* genes were expressed but beside the two wheat variants (scenario No. 3) also barley αCENH3 (but not βCENH3) was incorporated in all centromeres (Sanei et al. 2011). These data present overwhelming evidence for the predominance of scenario No. 3 (Figure 5) over a wide range of interspecific hybrids in the *Triticeae* tribe.

The cases of transgenic and native CENH3 combinations cannot be evaluated according to the listed scenarios because of the absence of parental relations. These reconstructed situations are suitable for establishing the boundaries of cross-species CENH3 loading in a homogeneous genetic background rather than for testing cross-specific incorporation into the centromeres in a hybrid genome. In addition, transgenic CENH3 proteins are usually detected indirectly via a large fluorescent protein tag, which is known to interfere with native CENH3 activity (Kalitsis *et al.*, 2003; Ravi *et al.*, 2011; Britt and Kuppu, 2016). It is therefore of no surprise that YFP-tagged maize CENH3 was not detected in the centromeres of transgenic wheat (Chen *et al.*, 2015) or wheat-maize somatic hybrids (Yang *et al.*, 2019).

The *α–* and *βCENH3* genes are the result of a gene duplication event, dated back to 35-40 million years ago (about the divergence time of the *Pooideae* subfamily from the *Oryzoideae* and *Panicoideae*), which modified the exon-intron structure of the original *CENH3* gene (Elisafenko *et al.*, 2021). At this time scale, specialisation and subfunctionalisation could have occurred between the CENH3 paralogues as demonstrated in cowpea in relation to generative development (Ishi *et al.*, 2020). The similar incorporation of the two wheat CENH3 proteins into barley centromeres in somatic as well as meiotic nuclei of the two wheat × barley hybrids points to no such functional deviation between the paralogous proteins as also observed in the non-hybrid background of barley (Ishii *et al.*, 2015b) and rye (Evtushenko *et al.*, 2021).

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 programmes. 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.

8 NEW SCIENTIFIC RESULTS

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

2. The presented research effectively developed and tested for the first time a palette of specific polyclonal antibodies suitable to detect three of the four 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. By combining molecular cytology with high resolution microscopy barley and wheat centromeric DNA was visualised simultaneously with the respective CENH3 protein variants in the three-dimensional structure of the mitotic and meiotic cell nuclei of wheat × barley F1 hybrids. This allowed to study endogenous and cross species incorporation of the different CENH3 histon 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 αCENH3 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 detetcted in meiotic cells and barley chromosome stability was confirmed during meiotic prophase I of the F1 hybrid.

9 CONCLUSION AND PROSPECTS

The combination of the genetic material of two closely related species via interspecific hybridisation is a key strategy to introduce new genetic diversity into cultivated crops and enhance crop resilience under the changing climate. Fertilisation between two related species generally results in male-sterile F1 hybrids that carry the haploid chromosome set of both parents. The reconstruction of the original diploid chromosomes set is required to restore fertility, which can be achieved via spontaneous or chemically induced genome duplication or via successive backcrossing and selfing. Incorporating agronomically desirable genes from barley to wheat via the generation of stable wheat × barley hybrid lines would provide a readily useable gene pool for future wheat breeding. A barrier to gene flow during wheat × barley hybridisation is selective chromosome elimination, which leads to the complete or partial loss of the barley chromosome sets at an early developmental stage. The present study elaborated the incorporation of parental CENH3 protein variants into the wheat and barley centromeres. It revealed the plasticity of barley centromeres in loading non-species specific CENH3 (i.e., wheat CENH3) in the hybrid nuclei. Moreover, the performed experiments showed that wheat and barley CENH3 proteins can co-habit within the same centromeres, without causing any apparent chromosome instability, at least during the mitotic cycles. This excludes an immediate effect of the ‘alien’ CENH3 protein incorporation in triggering chromosome instability and selective elimeination. An inefficient CENH3 loading, however, coincided with the elimination of the wheat genome in the hybrid No. 28/2020, where the entire barley chromosome complement was retained. This points to an indirect effect of CENH3, where unloading, or inefficient loading may be the result of other, possibly epigenetic reprogramming, ultimately leading to chromosome inactivity and mis-segregation. Further studies are needed to determine CENH3 loading in multiple genome combinations and through multiple generation to determine the factors triggering defective CENH3 protein loading. Understanding the process controlling centromere activity would open the way to directed chromosome retention or elimination, allowing to retain alien chromosomes with great agronomic interest and eliminate the unwanted alien chromosomes from the pre-breeding materials.

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12 SCIENTIFIC PUBLICATIONS

12.1 Peer-reviewed scientific papers

Polgári, D., Mihók, E. and Sági, L. (2019) Composition and random elimination of paternal chromosomes in a large population of wheat × barley (*Triticum aestivum* L. × *Hordeum vulgare* L.) hybrids. *Plant Cell Rep.,* **38,** 767–775.

Lenykó-Thegze, A., Fábián, A., Mihók, E., Makai, D., Cseh, A. and Sepsi, A. (2021) Pericentromeric chromatin reorganisation follows the initiation of recombination and coincides with early events of synapsis in cereals. *Plant J.*, **107**, 1585–1602.

Makai, D., Mihók, E.*, Polgári, D., Cseh, A., Lenykó-Thegze, A., Sepsi, A. and Sági, L. (2023) Rapid in-solution preparation of somatic and meiotic plant cell nuclei for high-quality 3D immunoFISH and immunoFISH-GISH. *Plant Meth.*, **19**, 80. (*co-first author)

Mihók, E., Polgári, D., Lenykó-Thegze, A., Makai, D., Fábián, A., Ali, M., Kis, A., Sepsi, A. and Sági, L. Plasticity of parental CENH3 incorporation into the centromeres in wheat × barley F1 hybrids. submitted

12.2 Conference proceedings

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13 SUMMARY

Climate change and its negative effects on agriculture make it necessary to develop new pre-breeding strategies seeking to widen the genetic diversity of bread wheat. Uniparental chromosome elimination in wheat × barley F1 hybrids is a major barrier to gene transfer from the barley genome to wheat. The aim of the present research was to better understand the biological processes underlying uniparental chromosome elimination, in order to abolish its effect in wheat × barley (and possibly other) hybrids.

We investigated the incorporation of parental CENH3 protein variants into wheat and barley centromeric DNA in primary hybrids. For this purpose we have developed a multi-step methodology whereby: 1.) an optimised root-tip nuclei preparation method was developed, allowing to rapidly produce a large number of high-quality microscopic preparations, without enzymatic treatment and complex manual tissue maceration. The optimised nuclear preparation is suitable for both separate and simultaneous use for *in situ* hybridisation and immunostaining. 2.) Wheat × barley F1 hybrids were produced and cytologically identified. 3.) Antibodies were designed and validated allowing the detection of species- and variant-specific CENH3 proteins from both parents within the somatic and meiotic nuclei of the hybrids. 4.) Specific DNA (FISH) probes were produced to enable the distinction of the two parental centromeric DNAs enclosed in the same hybrid interphase nuclei by fluorescent *in situ* hybridisation.

As a result, we were able to show for the first time that wheat α– and βCENH3 proteins can be incorporated into the centromeric DNA of barley in a partial wheat × barley F1 hybrid (No. 22/2020). We provided evidence that barley centromeres incorporate the barley αCENH3 variant, if it is available within the same nucleus. Thus, CENH3 proteins from both parents can co-localise within the same centromere and still remain functional in hybrid nuclei.

The analysis of the No. 28/2020 hybrid showed that, in contrast to the barley centromeres, wheat centromeric DNA could not incorporate (at least one of) the barley CENH3 proteins. Additionally, despite the presence of wheat CENH3 protein variants, unilateral and complete loss of wheat chromosomes was observed during mitotic cell divisions of the 28/2020 hybrid.

This study reveals, that further studies involving multiple hybrids and genome combinations are needed to understand the rules governing CENH3 incorporation through multiple generations. Understanding the factors that influence centromere

activity would open up the possibility to control the retention of individual chromosomes carrying the desired agronomical characteristics.

14 ÖSSZEFOGLALÓ

A klímaváltozás és annak a mezőgazdaságot sújtó hatásai szükségessé teszik olyan új elő-nemesítési stratégiák kidolgozását, amelyek a kenyérbúza genetikai sokféleségének kiterjesztésére törekednek. A búza-árpa F1 hibridekben lezajló uniparentális kromoszómaelimináció egyik fő gátja az árpa genomból búzába történő génátvitelnek. A jelen kutatás célja az uniparentális kromoszómaelimináció hátterében álló biológiai folyamatok közelebbi megértése, lehetővé téve azok kiiktatását.

Vizsgáltuk a szülői CENH3 fehérje variánsainak a búza és az árpa centromérikus DNS-be történő beépülését elsődleges hibridekben. A kutatás megvalósításhoz megfelelő, több lépcsős módszertan kidolgozására volt szükség: 1.) búza-árpa F1 hibrideket állítottunk elő, amelyeket citológiailag azonosítottunk 2.) A két szülő faj- és variáns specifikus CENH3 fehérjéinek egy hibrid sejtmagon belül történő kimutatásához saját tervezésű ellenanyagokat állíttattunk elő és validáltunk. 3.) Olyan specifikus fluoreszcens in situ hibridizációra (FISH) alkalmas DNS próbákat állítottunk elő, amelyek segítségével az interfázisos hybrid sejtmagokba zárt búza- és árpa szülői centromérikus DNS-ek egymástól megkülönböztethetővé váltak. 4.) Egy olyan sejtmag preparálási módszert optimalizáltunk, amelynek segítségével a korábbinál lényegesen gyorsabban, enzimkezelés és összetett manuális szövetfeltárás nélkül nagy mennyiségben állítható elő magas minőségű mikroszkópos preparátum. A kapott sejtmag preparátum alkalmas az *in situ* hibridizáció és az immunfestés külön-külön és egyidejű alkalmazására.

Eredményként először mutattuk ki, hogy a búza CENH3 fehérje α és β variánsa képes beépülni az **árpa** centromérikus DNS-ébe. Igazoltuk, hogy a búza CENH3 mellett az árpa centromérába beépül a sejtmagon belül rendelkezésre álló árpa αCENH3 variánsa is, így egy centromérán belül mindkét szülő CENH3 fehérjéi együttesen alakítják ki a centroméra funkcióját.

Meglepő módon, az árpa CENH3 fehérje hiányos bekötődése a búza centromérába, együtt járt a búza kromoszómák egyoldalú, és teljes kiesésével a 28/2020 hibrid mitotikus sejtjeiben. A fentiekből fakadó további kérdések megválaszolásához, a CENH3 bekötődésének további vizsgálataira van szükség, több genomkombináció bevonásával, és azok akár több generációban történő megfigyelésére. Ha megértjük a centroméra-aktivitást befolyásoló tényezőket, megnyílna a lehetőség az egyes

kromoszómák bennmaradásának illetve kiesésének irányítására, előidézve a hasznos agronómiai tulajdonságok örökítését távoli hibridekben.