HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

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Doctoral School of Plant Sciences

ESTABLISHMENT OF A TRACELESS GENOME EDITING SYSTEM IN WHEAT USING WHEAT X BARLEY HYBRIDIZATION

Doctoral (Ph.D.) dissertation

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The PhD Program

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1 INTRODUCTION AND OBJECTIVES

The rising global population has driven a 70% increase in cereal consumption, posing challenges to food security by 2050. Wheat, cultivated on more than 218 million hectares worldwide, contributes 20% of the global caloric and protein intake and ranks among the top three cereals, alongside rice and maize. However, wheat productivity is increasingly threatened by diseases and climate change. While selective breeding has enhanced traits in hexaploid wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD), limited recombination among sub-genomes restricts the use of its full allelic diversity.

Interspecific hybridization offers a promising approach to broaden genetic variation, particularly by introducing stress-resistance traits. Hybrids between wheat and barley (*Hordeum vulgare* L., 2n=2x=14, HH genome) can result in addition lines, translocations, or complete hybrids. However, reproductive barriers and frequent elimination of the paternal genome in *Triticeae* hybrids pose significant obstacles. While complete genome elimination facilitates maternal haploid production, partial hybrids serve as valuable materials for pre-breeding and trait introgression.

Genome editing technologies such as CRISPR/Cas9 provide precise tools for gene modification. This system introduces targeted double-strand breaks, repaired via non-homologous end joining (NHEJ) or homology-directed repair (HDR). Conventional delivery methods like *Agrobacterium*-mediated transformation are often inefficient in wheat and depend on genotype. Alternative approaches, including ribonucleoprotein delivery and the use of morphogenetic regulators, remain labor-intensive and genotype-dependent. Hi-Edit technology, which combines haploid induction with genome editing, is promising but still inefficient in wheat.

Hybridization with barley may enable DNA-free, CRISPR-based genome editing in wheat. After wheat × barley crosses, 20–90% of F1 hybrids are either haploid wheat or carry mixed chromosomes. Accurate characterization of these hybrids' chromosome composition is essential for further development.

This study aims to (1) evaluate the feasibility of DNA-free, CRISPR/Cas9-based mutagenesis via wheat × barley hybridization, and (2) develop a rapid,

cost-effective multiplex PCR system for determining chromosome composition in wheat x barley hybrids. The system's utility will be tested on diverse wheat and barley species, including wild relatives, to assess its broader applicability.

Objectives to achieve:

1. Establish a DNA-free genome editing platform for wheat by combining wheat × barley hybridization with CRISPR/Cas9 technology.
2. Generate F1 wheat × barley hybrid plants containing a CRISPR/Cas9 cassette to induce targeted mutations in the mlo allele of wheat.
3. Perform backcrossing of F1 hybrids with wheat to eliminate barley chromosomes and obtain transgene-free, edited wheat plants.
4. Develop a bioinformatic pipeline to analyze wheat and barley reference genomes and design chromosome-specific PCR primers for precise detection of individual chromosomes in wheat, barley, and their hybrids. This pipeline can be extended to other crops undergoing interspecific or intergeneric hybridization.
5. Establish and optimize a routine, fast, and cost-effective Multiplex PCR (MPCR) assay using these primers to assess chromosome composition in wheat, barley, their hybrid progeny, and related species within Triticum and Hordeum genera for trait improvement.

2 MATERIALS AND METHODS

2.1 Plant materials

For MPCR analysis and DNA-free genome editing, a doubled haploid wheat line ‘M1’ was crossed with barley ‘Golden Promise’. Plants were grown under controlled conditions in growth chambers, with synchronized flowering by staggered planting. Additional genotypes were grown in Jiffy peat pellets. ‘Golden Promise’ was also cultivated in phytotrons under optimum conditions to provide explants for *Agrobacterium*-mediated transformation.

2.2 Generation of CRISPR/Cas9 construct and barley transformation

We used the pHUE411 vector system (Xing et al., 2014), by inserting a 35S::DsRed gene from pC61KdsRED plasmid the pHUER plasmid was constructed. This plasmid contains a sgRNA targeting all three wheat homeoalleles of *TaMlo* gene. Immature barley embryos were transformed using *Agrobacterium tumefaciens* (strain AGL1) carrying the pHUER vector. Transgenic plants were identified by *DsRed* fluorescence and confirmed by PCR.

2.3 Crossing and embryo rescue

Wheat spikelets were trimmed and emasculated 2–3 days before anthesis. Spikes were bagged to prevent contamination. On flowering day, barley spikes were trimmed at the tip of each spikelet and put into warm water to induce anthesis. After pollination, a seed development inducing solution (SDIS) was injected into the internode to support caryopsis development. Fourteen days later, developing hybrid caryopses were harvested and embryos were isolated after surface sterilization (70% ethanol for 3 min, then 2% NaOCl for 20 min). Embryos were extracted by longitudinal dissection and germinated on N6D medium. If embryo numbers were low, they were cultured on callus induction, transition, and regeneration media.

2.4 DNA extraction

Total DNA was extracted from young leaf and root tissues using a direct method. Samples were placed in 100 µL Extraction Solution (E7526, Sigma-Aldrich) with a 3 mm stainless-steel bead (Qiagen) and homogenized in a mixer mill (Bullet Blender Storm Pro, Next Advance) at speed 8 for 1 min.

After heating at 95 °C for 12 min and cooling on ice for 1 minute, 100 µL of Dilution Solution (D5688, Sigma-Aldrich) was added. Samples were vortexed, centrifuged at 18,000 × g for 1 min, and the supernatant was taken and stored at 20 °C.

2.5 Generation of Multiplex PCR primers for the wheat and barley genomes

To initiate primer design, reference genomes of wheat (*Triticum aestivum* 'Chinese Spring', IWGSC RefSeq v1.0) and barley (*Hordeum vulgare* 'Golden Promise', GPv1) were retrieved from Ensembl and fragmented into all possible 20-mers using Jellyfish. These 20-mers were compared to those from three additional assemblies: wheat cultivars 'Weebill 1' and 'Claire', and barley 'Morex' (V3). Non-unique 20-mers repeated in any genome or matching in both orientations were discarded. Further filtering excluded 20-mers with fewer than three nucleotide types and those with less than 60% GC content. To identify reliable primer candidates, 100 random pairs per chromosome were mapped to the reference genomes using PatMaN, allowing up to two mismatches. Primers mapping more than once within a 1000-bp region were excluded. Amplified products were grouped into target size ranges (±5 bp): chr1 (100 bp), chr2 (150 bp), chr3 (200 bp), chr4 (250 bp, chr5 (300 bp), chr6 (350 bp), chr7 (400 bp). Primer pairs were pooled into plex-A, plex-B, and plex-D for the wheat sub-genomes, and plex-H for barley. To minimize off-targets, all primer sets were tested *in silico*, and those yielding high non-specific products were iteratively removed. Specificity was further verified by Ensembl BLAST. Where needed, primer positions were adjusted or extended. After individual PCR validation, each plex was tested *in silico* on 16 additional bread wheat assemblies, two barley assemblies, and genomes from wild and progenitor species to confirm robustness and broad applicability.

2.6 PCR and Multiplex PCR

Single PCR reactions were performed in 20 µL volumes using Phusion Hot Start II DNA Polymerase (F-549, Thermo Scientific) with 0.5 µM primers and 1 µL of direct DNA. Cycling: 98 °C for 3 min; 32 cycles of 98 °C for 10 s, 65 °C for 15 s, 72 °C for 10 s; final extension at 72 °C for 5 min. For *TaMlo* gene PCR, annealing was at 67 °C for 15 s over 30 cycles. Multiplex PCRs (MPCRs) targeting all wheat sub-genomes (A, B, D) and barley (H) were run with Phusion U Green Multiplex PCR Master Mix (F-564, Thermo

Scientific) using 0.3 µM primers. Cycling: 98 °C for 3 min; 32 cycles of 98 °C for 10 s, 65 °C (68 °C for plex-H) for 30 s, 72 °C for 10 s; final extension at 72 °C for 5 min. Variations using alternative enzymes (e.g., Phire Hot Start II, F-122) and buffers were tested with 68 °C annealing. All reactions were run on a Mastercycler® nexus gradient thermal cycler (Eppendorf, Hamburg, Germany).

2.7 Gel electrophoresis

PCR and MPCR products were separated on 1.2% and 2% (w/v) agarose gels, respectively, with ethidium bromide in 1X TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3). Gels were run at 120 V for 30 min (individual PCR) or 150 V for 30 min (MPCR). A GeneRuler™ 100 bp Plus DNA Ladder (Thermo Scientific) was used for size estimation. Gel images were captured using the ChemiDoc™ MP Imaging System (Bio-Rad), enabling evaluation of amplification success, primer specificity, and performance across genomes.

2.8 Genomic (GISH) and fluorescence (FISH) *In situ* hybridization

For the GISH-FISH analysis, the protocol described by Makai et al., (2023) was followed.

2.9 PCR amplicon purification and sequencing

PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) following the manufacturer's protocol. DNA concentration and purity were measured with a Nanodrop spectrophotometer (ThermoFisher Scientific). Amplicons from chromosomes 1A, 1B, 1D, and 1H were cloned into the pJET1.2/blunt vector (ThermoFisher Scientific) before sequencing due to their small size. Ligated vectors were transformed into *E. coli* DH5α via heat shock (42 °C, 45 s), and plasmids were extracted from colonies. Purified PCR products and plasmids were sequenced using respective primers by Eurofins Genomics (Germany).

2.10 Detection of targeted mutations

PCR products (3 µL) were digested in a 10 µL reaction with rCutSmart™ Buffer and Cac8I enzyme, incubated at 37 °C for 3 hours or overnight. Digested samples were run on a 1.2% agarose gel stained with ethidium bromide to detect mutations.

3 RESULTS AND DISCUSSION

3.1 Computational analysis and development of Multiplex PCR primers targeting specific chromosomes

To develop Multiplex PCR (MPCR) primer sets capable of specifically detecting individual chromosomes in wheat and barley, we began by breaking down two reference genomes into approximately 18.68 billion 20-base-pair (bp) sequences. These sequences were screened for uniqueness, sequence complexity, and GC content, to identify the most suitable primer candidates. The initial pool was reduced to 35.28 million high-quality sequences. We further selected sequences based on specific inter-primer distances. This refinement resulted in 270,413 primer pairs, which were then organized into four MPCR subsets: plex-A (targeting the wheat A sub-genome), plex-B (wheat B sub-genome), plex-D (wheat D sub-genome), and plex-H (barley genome). Despite successfully designing these primer sets, initial PCR testing revealed non-specific fragment amplification in several cases.

To address these shortcomings, we enhanced our protocol by incorporating additional genome data and refining filtering parameters. Specifically, we included three more genome assemblies (two wheat and one barley) to exclude sequences with non-unique or multiple occurrences. We also allowed mismatches and applied stricter controls for off-target and cross-off-target amplification. Through this improved screening process, the initial pool of 18.68 billion sequences was reduced to 287.29 million potential candidates with a maximum of two mismatches. Further filtering eliminated primer pairs prone to off-target amplification, and sequences were grouped by defined size ranges. From this refined set, we randomly selected 100 primer pairs per chromosome from the remaining 9,437 pairs.

Using *in silico* modeling, we simulated the use of all 700 primer pairs (per plex) in PCR experiments, allowing for two mismatches. Iterative refinement was performed by systematically removing primer pairs with the highest off-target amplification rates until a final cross-off-target-free set was achieved. The finalized primer sets underwent thorough validation through manual PCR testing and *in silico* cross-referencing against sequenced genomes from 16 bread wheat and two barley cultivars. A few anomalies were observed during validation. For example, primers

350bp_F_6A and 350bp_R_6A produced two distinct 346-bp amplicons from separate locations in the 'Robigus' wheat genome. This discrepancy may be attributed to incomplete genome assembly in this cultivar, potentially representing a bioinformatic artifact. In another case, primers 150bp_F_2B and 150bp_R_2B did not predict an amplicon in the genome of 'LRPB Lancer' wheat through bioinformatic analysis. However, PCR verification confirmed the expected product using DNA from this cultivar. Additionally, the primer pair 250bp_F_4D and 250bp_R_4D produced a 242-bp by-product from chromosome 4B from wheat cultivars 'LRPB Lancer', 'Paragon', 'SY Mattis', and 'Julius'. Sequence analysis revealed a mismatch at the 3' end of the 250bp_F_4D primer, which likely disrupted amplification, as corroborated by sequencing data.

The evolutionary context helps explain some of these anomalies. The homology between hexaploid wheat and barley estimated to be in the range of 45% to 60%. This sequence similarity creates both opportunities and challenges for developing species-specific marker sets. On the one hand, homology allows for the design of primers that can distinguish between wheat and barley chromosomes, but on the other hand, it also introduces the risk of off-target amplifications or cross-reactions.

3.2 Assessment of the designed MPCR primer sets

The amplicons generated by the designed MPCR (Multiplex PCR) primer sets were specifically tailored to produce distinct product sizes, increasing stepwise for each chromosome across the (sub-)genomes. These incremental sizes were strategically assigned as follows: chr1 – 100 bp, chr2 – 150 bp, chr3 – 200 bp, chr4 – 250 bp, chr5 – 300 bp, chr6 – 350 bp, and chr7 – 400 bp. This systematic size progression ensured clear differentiation of MPCR products during electrophoretic separation, simplifying downstream analysis and interpretation.

To comprehensively verify the specificity and sensitivity of the designed primer pairs, we first conducted individual PCR tests using DNA templates derived from reference genomes. The results showed precise amplification of products corresponding to the expected sizes in all tested scenarios (Figure 1A). Each primer set consistently generated a single, well-defined amplicon without any nonspecific by-products, indicating their high target affinity and amplification efficiency.

In particular, the wheat-specific primers, derived from the cultivar ‘Chinese Spring,’ successfully detected their corresponding wheat chromosomes with remarkable precision. Simultaneously, the barley-specific primers demonstrated no cross-reactivity with wheat genomic DNA, further emphasizing their species-specificity (Figure 1A). To validate these results at the molecular level, we performed sequence analyses on all amplified products. These analyses confirmed that each of the 28 PCR products matched the sequences predicted through *in silico* bioinformatic analysis. This correlation between experimental and computational data provides robust evidence that the primer sets are highly reliable, with no off-target amplification events.se

Following these initial verifications, we evaluated the performance of the chromosome-specific primer sets in a more complex MPCR setup using total genomic DNA extracted from two widely studied reference cultivars: ‘Chinese Spring’ (wheat) and ‘Golden Promise’ (barley). In the Multiplex reaction setup, primer sets were grouped according to their corresponding (sub-) genomes, and the results were analyzed based on their chromosomal origin. The MPCR experiments (Figure 1B) yielded clear, distinct bands for all target chromosomes from both wheat and barley genomes. Importantly, the size of the amplified products was consistent with those observed in the single PCR tests (compare Figure 1A and Figure 1B), reaffirming the reproducibility and accuracy of the MPCR assay.

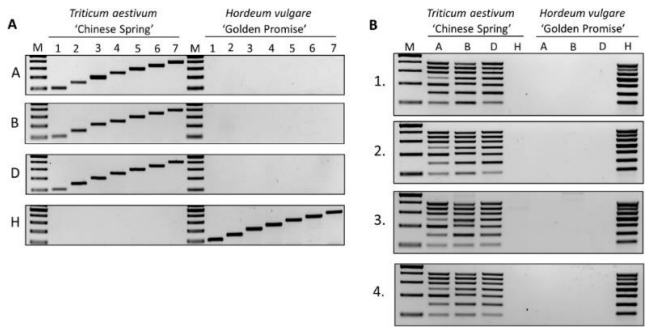

Figure 1: A: Single PCR amplifications were performed using primers specific to chromosomes 1–7 from the A, B, and D sub-genomes of wheat (‘Chinese Spring’) and the H genome of barley (‘Golden Promise’). Lane M shows the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). **B:** MPCR amplification was conducted for chromosomes 1–7 of the A, B, and D sub-genomes of wheat (‘Chinese Spring’) and the H genome of barley (‘Golden Promise’) using various buffer and DNA polymerase combinations to evaluate protocol flexibility (Ali et al., 2024).

A critical observation from the MPCR experiments was the absence of nonspecific products and lack of cross-reactivity between wheat and barley DNA. To assess the technical flexibility and robustness of the MPCR protocol, we further tested the method using four commercially available buffer-DNA polymerase systems. All four supported successful DNA amplification without introducing nonspecific products (Figure 1B). This indicates that the MPCR protocol is adaptable to a range of commonly used commercial reagents, making it highly versatile for laboratories with varying resources and preferences.

The MPCR assay was designed to amplify chromosome-specific loci from each wheat sub-genome (A, B, and D) and from barley (H genome) in multiplex reactions. This allowed multiple chromosomes to be detected in a single reaction, improving throughput and reducing time and cost compared to cytogenetic techniques like GISH and FISH.

3.3 Broad applicability of MPCR across a wheat and barley panel

Total DNA samples were extracted from 14 wheat cultivars and five barley cultivars and analyzed through separate MPCR reactions targeting the wheat A, B, and D sub-genomes as well as the barley H genome (Figure 2: panels A to H, respectively). These MPCR reactions yielded distinct and well-defined band patterns corresponding to the expected sizes for all (sub-) genomes. The specificity of the primer sets was confirmed by the clear and consistent amplification of the target regions, with no non-specific cross-reactions observed across the different wheat and barley cultivars.

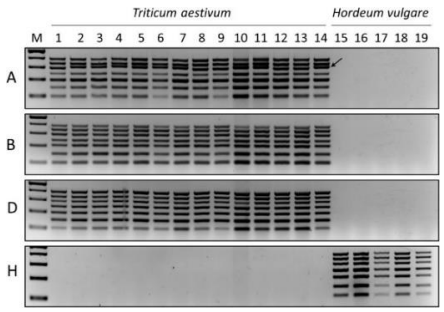

Figure 2: MPCR amplification was conducted on chromosomes 1–7 of the A, B, and D sub-genomes from 14 wheat cultivars and the H genome of five barley cultivars. One notable finding indicated with arrow, an increased size of the 5A-specific product in several wheat cultivars. The molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder) is denoted by M, (Ali et al., 2024).

One notable observation was the minor variation in the size of the 5A chromosome-specific amplicon in several wheat cultivars, including 'Bobwhite,' 'Fielder,' 'Bánkúti 1201,' 'LRPB Lancer,' 'CDC Stanley,' 'Paragon,' 'Cadenza,' 'Weebill 1,' and 'Jagger' (Figure 2: panel A, arrow). While the amplification was still specific, a slight increase in product size was noted in these cultivars. Further *in silico* sequence analysis of these particular amplicons revealed that they contained a 12-bp insertion within the corresponding genomic region. This insertion was consistent across all the affected cultivars, confirming that the size discrepancy was due to a genomic variation rather than primer inefficiency or non-specific amplification.

3.4 MPCR primer sets effectively determine the chromosome composition of wheat × barley hybrids.

As a practical application of the designed MPCR primer sets, we evaluated their ability to assess the chromosome composition in 'M1' wheat (♀) × 'Golden Promise' barley (♂) F1 hybrid plants. A total of 16 hybrid plants were regenerated from 18 embryos rescued from 20 pollinated spikes. MPCR analyses of the plants revealed that they all contained the complete set of wheat chromosomes, with one exception: plant No. 14, which exhibited a faint band for the 3B wheat chromosome-specific product (Figure 3). This suggests a slight variation or incomplete amplification for this specific chromosome in this plant, but the overall wheat chromosomal composition was maintained in the majority of plants.

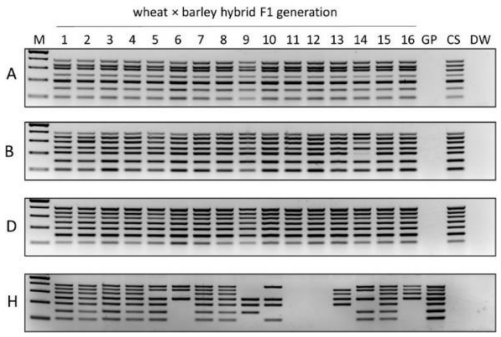

Figure 3: MPCR amplification was performed on chromosomes 1–7 of the A, B, D, and H sub-genomes from wheat × barley hybrids (1–16). M represents the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP refers to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW represents the no-template control (Ali et al., 2024).

In addition to wheat chromosomes, the barley chromosome composition was also assessed. Seven of the 16 hybrid plants (Nos. 1–4, 7–8, and 15) exhibited the presence of all barley chromosomes in their MPCR profiles, indicating that these plants successfully retained the full barley genome. However, the remaining hybrid plants exhibited varying degrees of chromosome retention: three plants (Nos. 6 and 9) retained five barley chromosomes, while four plants (Nos. 10, 13, and 16) had four barley chromosomes, and one plant (No. 14) retained only three barley chromosomes. The data show that barely chromosomes were maintained and not lost during early development. Notably, two plants (Nos. 11 and 12) were identified as maternal wheat haploids, as evidenced by the complete absence of barley chromosomes in their MPCR profiles.

To validate the effectiveness and accuracy of our MPCR technology, we selected two hybrid plants (Figure 11: Nos. 6 and 13) for further analysis using GISH (Genomic *In Situ* Hybridization) and FISH (Fluorescence *In Situ* Hybridization) techniques, employing a barley 5S rDNA-specific probe to identify individual barley chromosomes (Figure 3A and B). Since mitotic chromosomes are typically obtained from root tips, we selected two root tips from each hybrid plant to be processed simultaneously for both *in situ* hybridization and MPCR analysis, allowing us to directly compare the results from both methods.

The GISH-FISH analysis of hybrid plant No. 6 revealed the presence of barley chromosomes 4H + 5H in one root tip, and 4H + 6H + 7H in the other root tip (Fig. 3A). These findings were in full agreement with the results obtained from the MPCR analysis (Fig. 3C), confirming that plant No. 6 is genetically mosaic, with different root tips containing different barley chromosome combinations. Similarly, for hybrid plant No. 13, GISH-FISH analysis showed that both root tips contained barley chromosomes 3H–6H (Fig. 3A), which was also consistent with the MPCR results (Fig. 3C). These parallel results from GISH-FISH and MPCR analysis indicated that the distribution of barley chromosomes in plant No. 13 was uniform across both root tips.

Unlike GISH and FISH, which require microscopy and detailed cytological preparation, MPCR is a fast, high-throughput method that can be applied to large sample sets. It is particularly valuable for F1 hybrids, where intact

chromosomes can be easily detected. In later generations with recombination, MPCR can complement cytogenetic methods. MPCR also detects genetic mosaicism more effectively than cytogenetic methods. Since DNA is extracted from many cells, MPCR can reveal mixed chromosome populations that might be missed when only a few cells are examined microscopically.

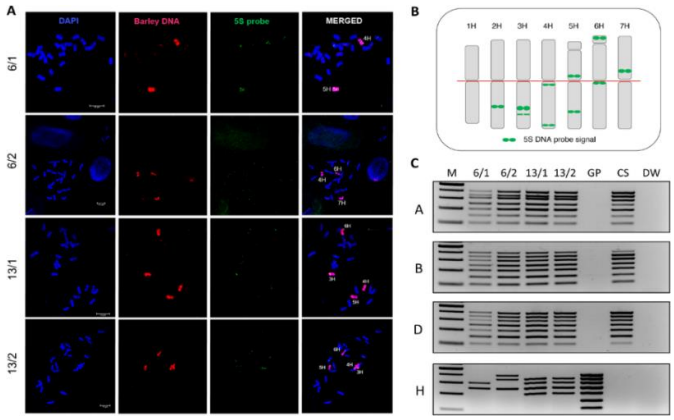

Figure 3: A. Chromosome in situ hybridization was performed on root segments from two wheat × barley hybrids (plants 6 and 13; Fig. 4). Barley chromosomes were visualized using GISH (red) and identified with FISH using a 5S rDNA probe (green), with DAPI counterstaining (blue); scale bars = 10 µm. **B.** A schematic shows the 5S rDNA probe location on the barley genome, with the centromere marked in red. **C.** MPCR amplification of chromosomes 1–7 from A, B, D, and H sub-genomes (plants 6 and 13) was performed using DNA from the same roots as GISH (Ali et al., 2024).

3.5 MPCR analysis of closely related *Triticum* and *Hordeum* species

To assess the broader applicability of the primers, we tested them on wild relatives and progenitor species of wheat and barley to understand their taxonomic limits. The *in silico* alignment of the final primer set with the available seven sequenced genomes showed perfect homologies and no unspecific products in the genome sequences of *T. spelta* (AABBDD sub-genomes), *T. turgidum* ssp. durum (AABB), *T. dicoccoides* (AABB), *Aegilops tauschii* (DD), and *H. spontaneum*. These results confirmed the high specificity of the primers for these species. However, alignment with the genomes of *T. urartu* (AA) and *H. marinum* (XaXa) revealed the absence of several chromosome-specific target sites. Specifically, only four of the seven chromosome-specific target sites were found in *T. urartu*, and only two were present in *H. marinum*, with these sites marked in red.

In contrast to the predictions made from the *in silico* analysis, the MPCR results did not completely align with expectations for *Triticum* and *Hordeum* species (Figure 4). In *T. spelta*, a hexaploid species, the 7B chromosome-specific primer pair failed to amplify any product, a result also observed in the tetraploid *T. dicoccoides* (AABB), although successful amplification occurred in *T. dicoccum* (also AABB). Similarly, in *T. turgidum* ssp. durum (AAABB), the 6A chromosome-specific product was absent (Figure 4A: top panel). These discrepancies can be attributed to several factors, including the genetic diversity present within the wild species and the fact that some of the species tested may not have been fully represented by the reference genome sequences. However, when these two problematic primer pairs were replaced with new ones, the correct products were successfully amplified (Figure 4B), showing that alternative primers can be an effective solution for these species.

The MPCR results with the A-genome species *T. monococcum* and *T. urartu* produced partial plex-A-specific patterns, with four and five products, respectively (Figure 4A: middle panel). Additionally, some unspecific products were generated with the plex-B primers. These results suggest that while the primers are generally applicable, some species may show partial amplification patterns due to genomic differences or primer-template mismatches.

Interestingly, in *Ae. speltoides*, the hypothesized donor of the B sub-genome, up to six correct bands were obtained with the plex-D primers, along with six faint bands from the plex-B primers and some correct-sized products from the plex-A primers as well (Figure 4A: middle panel). In contrast, *Ae. tauschii*, the definitive D-genome donor, also produced some bands with the plex-A and plex-B primers, further illustrating the genomic complexity of wheat and its wild relatives.

Finally, the plex-H primers, designed for barley chromosomes, worked as expected with *H. spontaneum* (HH genome), the closest relative of cultivated barley. However, when tested on more distant relatives such as *H. bulbosum* (HbHb) and *H. marianum*, the results were partial, with fewer bands observed in these species (Figure 4A: bottom panel). This suggests that while the primers are effective for barley, their performance may vary across species with more distant evolutionary relationships.

These findings are consistent with the known evolutionary history of hexaploid wheat. Hybridization events between A- and B-genome ancestors around 3–4 million years ago contributed to the formation of the D-genome lineage, now represented by *Ae. tauschii*. This polyphyletic origin introduced sequence overlaps among the A, B, and D genomes. As a result, cross-reactivity of primers and reduced precision in some wild species are expected. The complexity of wheat's evolutionary background complicates the design of truly chromosome-specific primers, particularly for the D-genome, and is reflected in occasional mismatches and cross-amplifications observed in the MPCR analysis of wild relatives.

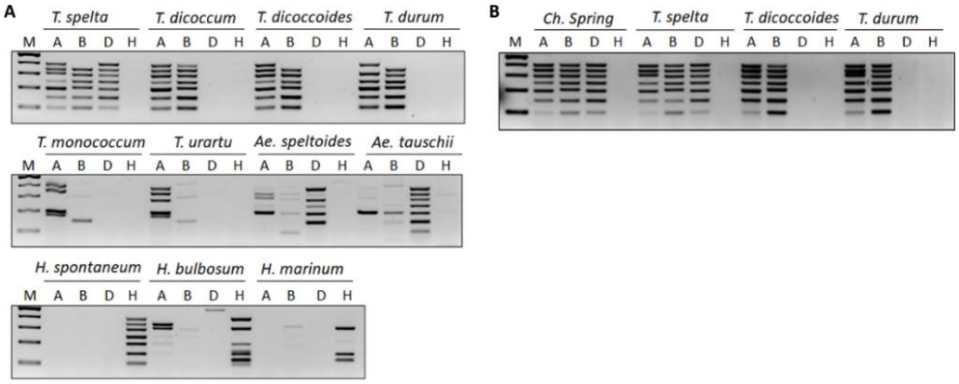

Figure 4: A. MPCR amplification of chromosomes 1–7 from the A, B, and D sub-genomes of wheat and the H genome of barley, using various wheat and barley species. **B.** Enhanced MPCR amplification with substituted primer pairs specific for the 6A and 7B chromosomes. M – Molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder) (Ali et al., 2024).

3.6 Transformation of barley with CRISPR/Cas9 vector

To justify our hypothesis of mutation creation in the wheat *TaMlo* gene via transgenic barley pollen, we identified the sequences of all three *MLO* homeoalleles (5A-TraesCS5A02G494700, 4D-TraesCS4D02G319000, and 4B-TraesCS4B02G322600) in 'Chinese Spring'. To obtain transformed barley lines, a designed sgRNA targeting all three homeoalleles was selected. *Agrobacterium*-mediated transformations of 150 immature barley embryos with the pHUER vector construct containing Tamlosg2 were carried out. Ten independent lines from three different calli were selected based on DsRED expression. For further confirmation, PCR was carried out on the hygromycin gene (*hptII*) to verify the presence of the transgene. For crossing, only one plant from the T0 generation was selected and self-

pollinated to produce the T1 generation. To check the heritability of the transgene, the selected T1 plants were further propagated to the T2 and T3 generations. It was found that in the selected representatives, the transgene did not show segregation.

3.7 Chromosome Composition and Mutation Analysis in F1 Hybrids

During the first crossing, we produced 37 F1 hybrid embryos. The embryos were examined under a light microscope at a wavelength of 550 nm to assess DsRED expression, and some embryos exhibited DsRED fluorescence (Figure 5E). Following DsRED expression analysis, the embryos were directly germinated on N6D medium. The regenerated plants were initially subjected to chromosome composition analysis using our MPCR-based chromosome composition detection marker system. All the F1 hybrids contained full wheat chromosomes, and the barley chromosomes were present in random numbers. Plants 2 to 12, along with 15, 16, 28, and 32, were identified as full hybrids. Plants 19, 24, 26, 27, 31, and 36 completely lacked barley chromosomes. Meanwhile, plants 1, 13, 14, 17, 18, 21, 22, 23, 25, 29, 30, 33, 34, 35, and 37 contained random numbers of barley chromosomes. Additionally, alongside H-genome detection primers, the Cas9 gene was amplified, as indicated by black arrows (Figure 5).

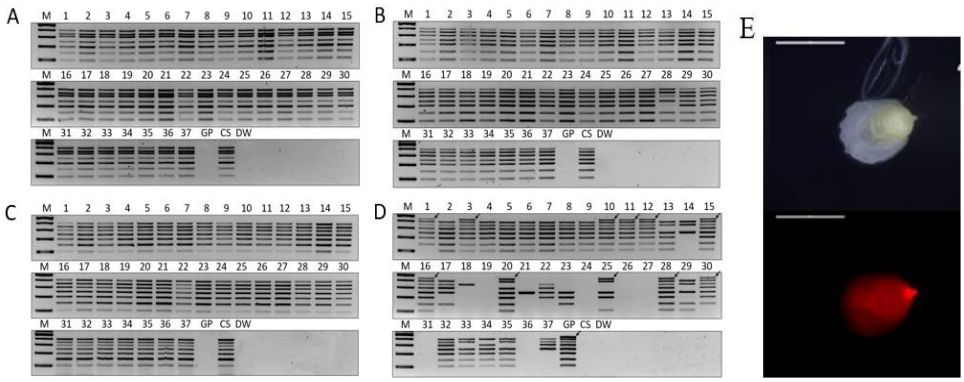

Figure 5: A. MPCR amplification of chromosomes 1–7 from the A sub-genome **B.** MPCR amplification of chromosomes 1–7 from the B sub-genome. **C.** MPCR amplification of chromosomes 1–7 from the D sub-genome. **D.** MPCR amplification of chromosomes 1–7 from the H genome. Cas9 positive plants are indicated with black arrows. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP corresponds to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW is the no-template control. **E.** A hybrid embryo showing expression of DsRED under 550 nm wavelength (Lower picture) and same embryo at day light (Upper picture).

The PCR/RE assay shown in the image (Figure 6) was conducted to analyze mutations in the wheat *TaMlo* gene using specific primers, followed by digestion with the restriction enzyme *CaC*8I. Wild-type *Mlo* alleles are expected to be fully digested, producing two distinct fragments, while mutant alleles lacking the *CaC*8I recognition site remain undigested. PCR amplicons from plants 10, 11, 15, 16, 20, 25, and 28 were not completely digested, suggesting the presence of mutations. Mutated plants are indicated with red arrows.

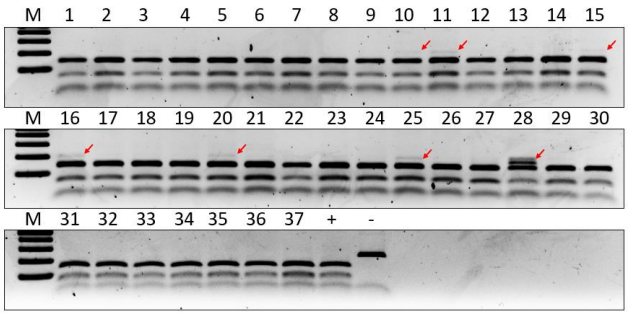

Figure 6. PCR/RE assay to detect mutation in F1 plants. *TaMlo* gene amplified using specific primers and digested with *CaC*8I restriction enzyme. Mutated plants are indicated with red arrows. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). (+) sample is PCR amplicon from wild type 'Chinese Spring' with enzyme and (-) sample is WT without enzyme.

3.8 Chromosome Composition and Mutation Analysis in F1BC1 Hybrids

In an attempt at backcrossing all, F1 hybrids containing any barley chromosomes were backcrossed and successfully 16 backcrossed plants were produced. Most of the time one spike but sometimes 2 spikes per plant were backcrossed. Like the F1 hybrids, these plants were first subjected to chromosome composition analysis using the MPCR-based chromosome detection marker system. All plants contained full wheat chromosomes. As with the F1 hybrids, plants 6, 7, 11, 12, 14, and 16 were identified as aneuploids with two sets of wheat chromosome from maternal and paternal side but barley chromosomes can be only inherited from maternal side, while plants 1 and 8 lacked all barley chromosomes. These plants were fertile but do not have mutation. Plant 13, which has only 4H chromosome, was also fertile but not mutated. The remaining plants contained barley chromosomes in random numbers. Plant 9 lacks the barley chromosome 5H entirely. Karyotyping analysis suggests it likely retains two copies of all wheat

chromosomes, while possessing single copies of the remaining barley chromosomes (1H, 2H, 3H, 4H, 6H, and 7H). This plant was mutated but sterile. All other plants which have barley chromosomes were sterile and could not survive. Additionally, the Cas9 gene was amplified along with barley chromosomes, as indicated by black arrows (Figure 7).

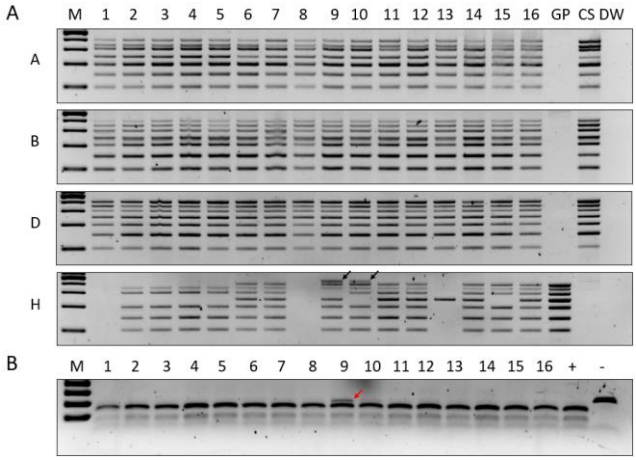

Figure 7: A. MPCR amplification of chromosomes 1–7 from the A, B, D sub-genome of wheat and H genome of barley. Cas9 positive plants are indicated with black arrows. **B.** PCR/RE assay to detect mutation in F1BC1 plants. *TaMlo* gene amplified using specific primers and digested with *CaC*8I restriction enzyme. Mutated plant is indicated with red arrow. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP corresponds to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW is the no-template control. (+) sample is PCR amplicon from wild type 'Chinese Spring' with enzyme and (–) sample is WT without enzyme.

3.9 In vitro propagation of F1 embryos

In continuous efforts of crossing where fewer F1 hybrid embryos are available, they can be propagated through callus induction. In one instance, we propagated 35 plants from one embryo. These plants were first subjected to chromosome composition analysis. As shown in (Figure 8), all 35 plants contained full wheat chromosomes for the A, B, and D sub-genomes. Regarding H chromosomes, the 6H chromosome of barley was missing from all plants, which can be expected since all these plants were propagated from a single embryo. Additionally, in plants 28 and 31 along with 6H, the 1H and 2H chromosomes were also missing, respectively.

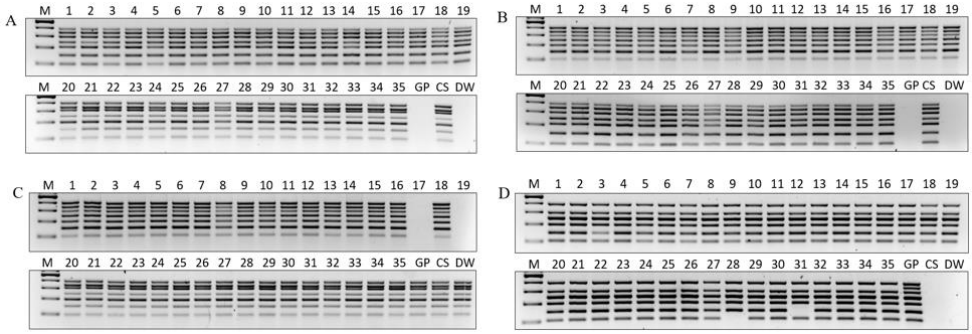

Figure 8: A. MPCR amplification of chromosomes 1–7 from the A sub-genome **B.** MPCR amplification of chromosomes 1–7 from the B sub-genome. **C.** MPCR amplification of chromosomes 1–7 from the D sub-genome. **D.** MPCR amplification of chromosomes 1–7 from the H genome. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP corresponds to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW is the no-template control.

After chromosome composition analysis, mutation analysis was carried out using a PCR/RE assay. The *TaMlo* gene was amplified with specific primers. Following amplification, PCR products were digested with the *CaC*8I enzyme. Mutated plants are indicated with red arrows (Figure 9). PCR/RE assay results revealed varying levels of mutations among the analyzed plants, as indicated by the intensity of undigested PCR amplicons. While most plants exhibited low levels of mutation, as seen in faint undigested bands (e.g., Plants 13, 15, 18, 19, 22, 24 and 28), But plant 32 displayed significantly higher mutation level, evidenced by prominent undigested bands.

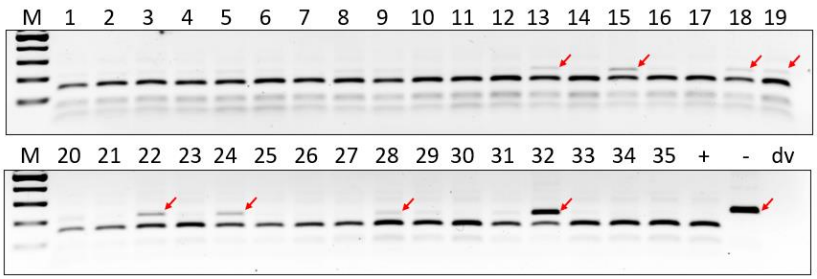

Figure 9. PCR/RE assay to detect mutation in F1 propagated plants. *MLO* gene amplified using specific primers and digested with *CaC*8I restriction enzyme. Mutated plants are indicated with red arrows. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). (+) sample is PCR amplicon from wild type 'Chinese Spring' with enzyme and (–) sample is WT without enzyme.

3. 10 Cloning of F1 plants via immature inflorescence

To clone plant 32, which shows high degree of mutation, immature inflorescences approximately 1–2 cm in size was sterilized and placed on callus induction medium (Figure 10). Successfully, 28 plants were cloned. When these plants were analyzed for chromosomes composition, all these plants contain all 7 wheat chromosomes for A, B, D and H sub-genomes. And they retain the same barley chromosome composition. They have 1 to 7 chromosomes of barley except 6H chromosome was missing in all these plants. Which is expected because they are cloned from a single inflorescence. After chromosome composition analysis, the *MLO* gene was amplified. Following amplification, PCR products were digested with the *CaC*8I enzyme. After analyzing these plants for mutations using the PCR/RE assay, most plants exhibited mutations with nearly identical intensities of digested fragments. However, some plants displayed varying levels of mutation, as evidenced by differences in the intensity of undigested PCR amplicons.

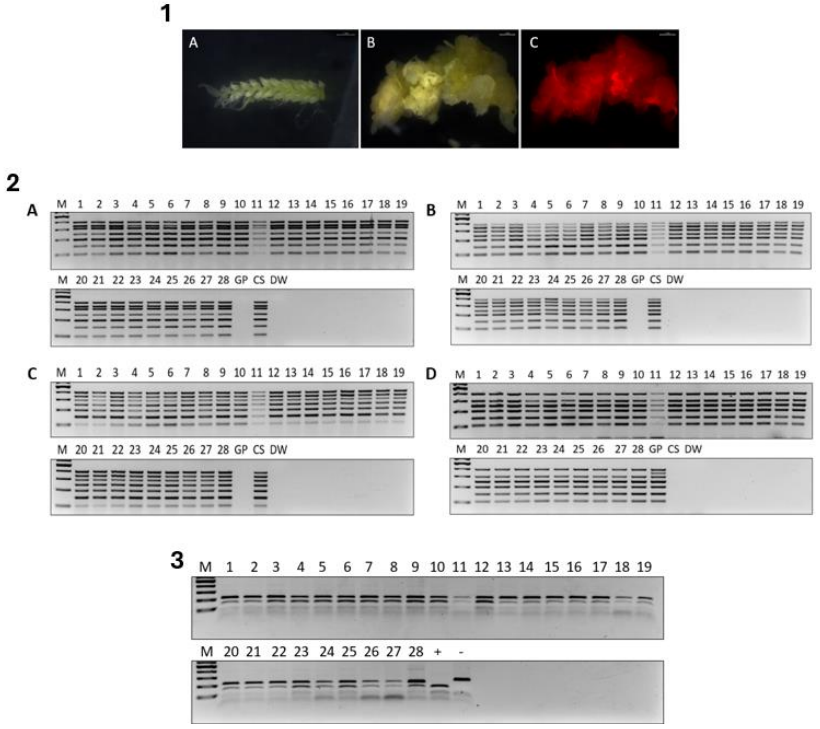

Figure 10. 1. Immature inflorescence tissue was placed on a callus induction medium to facilitate the generation of clonal plants. **A.** Immature inflorescence excised from the plant. **B.** Formation of yellowish callus tissue after successful induction. **C.** Fluorescent image showing DsRed expression in the callus, confirming the presence of the transgene in the induced tissue. **2. A.** MPCR amplification of chromosomes 1–7 from the A sub-genome **B.** MPCR amplification of chromosomes 1–7 from the B sub-genome. **C.** MPCR amplification of chromosomes 1–7 from the D sub-genome. **D.** MPCR amplification of chromosomes 1–7 from the H genome. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP corresponds to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW is the no-template control. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase. **3.** PCR/RE assay to detect mutation in immature inflorescence propagated plants. *MLO* gene amplified using specific primers and digested with *CaC*8I restriction enzyme. Sample 1 to 28 are PCR amplicons from cloned plants. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). (+) sample is PCR amplicon from wild type 'Chinese Spring' with enzyme and (–) sample is WT without enzyme. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase.

4. CONCLUSION AND RECOMMENDATIONS

This study presents an optimized Multiplex PCR (MPCR) assay for identifying individual chromosomes and monitoring their composition in wheat × barley hybrids. Using wheat and barley reference genomes, large primer sets were designed to target chromosome-specific regions. These primers were validated *in silico* against 18 cultivars and tested experimentally on 19 wheat and barley cultivars, as well as 11 species from the genera *Triticum*, *Aegilops*, and *Hordeum*, confirming their specificity and robustness. The successful application of the MPCR assay on 16 wheat × barley F1 hybrids, with results corroborated by the traditional GISH technique, validated the method's efficiency for chromosome-specific locus detection.

This approach holds strong potential for crop breeding and genetic research, providing a scalable method to monitor chromosome composition in hybrids. The primer design strategy is adaptable to other species with sequenced genomes. Rapid, accurate chromosome tracking can accelerate breeding for traits like disease resistance, drought tolerance, and higher yield. The MPCR assay also offers a high-throughput, faster alternative to traditional cytogenetic methods, enhancing F1 hybrid characterization and karyotyping. The use of Multiplex Polymerase Chain Reaction (MPCR) has emerged as a transformative tool in accelerating plant breeding by enabling precise and efficient identification of genetic variations across complex plant genomes. Studies, such as Koh et al. (2017) for distinguishing Brassica species, highlight its application in polyploid crops, demonstrating cost-effectiveness and genome specificity. By optimizing primer sets for diverse genetic backgrounds, MPCR methods can enhance the sensitivity and robustness of assays, ultimately accelerating the development of crops with improved traits.

Moreover, integrating the MPCR assay with technologies such as fluorescence *In situ* hybridization (FISH) could provide a more comprehensive understanding of chromosome integrity, translocation, and other structural rearrangements. These complementary technologies would allow for a more thorough genomic analysis, helping researchers detect subtle genetic changes that may not be fully captured by MPCR alone.

Sequencing efforts for wild species and progenitors of wheat, barley, and other crops are essential to enhancing the MPCR assay's reliability. By increasing the number of sequenced accessions, researchers will gain richer genomic data, leading to the development of more accurate and comprehensive primer sets. This will be particularly important for addressing the high genetic diversity found in wild species, improving the accuracy of the MPCR assay for species with less well-characterized genomes. As genome sequencing becomes increasingly accessible, continuous refinement and validation of the MPCR primer sets will be essential to ensure the assay's adaptability and accuracy. Expanding the assay's application to a greater number of plant species and ensuring consistency across different cultivars will cement its place as a transformative tool in plant genetic research. The MPCR assay developed in this study represents a fast, cost-effective, and efficient method for analyzing chromosome composition and structure, with wide-ranging implications for plant breeding, hybrid characterization, and genetic studies.

On other hand the DNA free genome editing study represents a significant advancement in applying CRISPR/Cas9-mediated genome editing to wheat using transgenic barley pollen. By targeting all three homeoalleles of the wheat *TaMLO* gene with a carefully designed sgRNA, we demonstrated the feasibility of inducing mutations in wheat through hybridization. The successful generation of F1 hybrids expressing the Cas9 system underscores the potential of barley pollen as a novel transgene delivery system for wheat genome editing. However, challenges such as the inconsistent and low mutation efficiency in some F1 hybrids, and reduced fertility in F1BC1 hybrids were observed. These limitations highlight the need for further optimization to fully exploit this innovative method. Despite these challenges, the study provides a robust proof of concept for the delivery of transgenic pollen having CRISPR/Cas9 system in wheat via wheat x barley hybridization to facilitate genetic improvement in polyploid crops like wheat.

Increasing the mutation efficiency in hybrid plants remains a critical goal. Codon optimization of the Cas9 gene, designing more effective sgRNAs, or targeting less complex genomic loci could improve mutation rates across generations.

Hypothetically, the presence of unpaired barley chromosomes in F1BC1 hybrid plants poses a challenge because it causes infertility. Although literature suggests that several additional lines exhibit disomic addition and remain fertile, it is also true that a full amphiploid has not been achieved despite numerous attempts. Future studies should focus on improving hybrid fertility, which is paramount for scaling this approach. Advanced mutation detection techniques, such as next-generation sequencing, should complement traditional PCR/RE assays to provide a more comprehensive understanding of editing outcomes. Finally, refining *in vitro* propagation techniques for F1 hybrid embryos can enhance the consistency of chromosome composition and mutation frequency, facilitating the large-scale generation of F1 edited plants. Propagation of F1 plants through immature inflorescence is another option to clone and get large number of edited plants with almost consistent mutations. By addressing these recommendations, future research can unlock the full potential of CRISPR/Cas9-mediated genome editing in wheat through transgenic barley pollen.

5. NEW SCIENTIFIC RESULTS

- A bioinformatics pipeline was designed to identify chromosome-specific primers, focusing on wheat and barley genomes, which can be applicable for other species.
- A novel, fast, and cost-effective MPCR-based technology was developed to assess chromosome composition of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and their hybrids.
- Additionally, the primer sets are compatible with various wheat and barley cultivars and effective on closely related *Triticum* and *Hordeum* species.
- The MPCR methods is suitable for chromosome detection from any plant tissue, unlike the *in situ* techniques (GISH, FISH).
- Mutations were created in wheat *MLO* gene via wheat x barley hybridization.
- Mutated F1 plants can be propagated on callus induction medium through immature inflorescence.
- The barley genome carrying the transgene can be removed from the hybrid by backcrossing with wheat.

6. PUBLICATIONS LIST

Conference Presentations and Posters:

Ali, M., Polgári, D., Sepsi, A., Kontra, L., Dalmadi, Á., Havelda, Z., Sági, L., & Kis, A. (2024, June 16–19). *Fast and effective MPCR-based molecular karyotyping in wheat, barley, and their cross-progeny* [Poster presentation]. EMBO Workshop: Plant Genome Stability and Change, Palacký University, Olomouc, Czech Republic.

Ali, M., Polgári, D., Sági, L., Havelda, Z., & Kis, A. (2023, November 30–December 1). *Establishment of powdery mildew resistance in wheat with CRISPR/Cas9 technology* [Oral presentation]. Genetics and Biotechnology Institute Days, Gödöllő, Hungary.

Ali, M., Polgári, D., Sepsi, A., Kontra, L., Sági, L., Dalmadi, Á., Havelda, Z., & Kis, A. (2023, May 15–20). *Rapid and cost-effective genotyping in wheat × barley hybrids by chromosome-specific multiplex PCR* [Oral presentation]. European Association for Research on Plant Breeding – Cereal Section Conference, Cereal Research Nonprofit Ltd. (GK), Szeged, Hungary.

Ali, M., Polgári, D., Kontra, L., Sági, L., Dalmadi, Á., Havelda, Z., & Kis, A. (2022, April 11–12). *A traceless CRISPR/Cas9 approach for precision mutagenesis in bread wheat (Triticum aestivum L.) via distant hybridization*. In *FIBOK 2022: 5th National Conference of Young Biotechnologists* (pp.86). Gödöllő, Hungary. ISBN 978-963-269-999-8.

Kis, A., Polgári, D., **Ali, M.,** Sági, L., & Havelda, Z. (2021, December 14). *Hi-Breeder: A new tool for efficient "DNA-free" wheat (Triticum aestivum L.) genome editing* [Oral presentation]. Genetics and Biotechnology Institute Days Conference, Gödöllő, Hungary.

Publications:

Ali, M., Polgári, D., Sepsi, A., Kontra, L., Dalmadi, Á., Havelda, Z., Sági, L., & Kis, A. (2024). Rapid and cost-effective molecular karyotyping in wheat, barley, and their cross-progeny by chromosome-specific multiplex PCR. *Plant methods*, *20*(1), 37. https://doi.org/10.1186/s13007-024-01162-x

Mihók, E., Polgári, D., Lenykó-Thegze, A., Makai, D., Fábián, A., **Ali, M**., Kis, A., Sepsi, A., & Sági, L. (2024). Plasticity of parental CENH3 incorporation into the centromeres in wheat × barley F1 hybrids. *Frontiers in Plant Science*, *15*, 1324817. https://doi.org/10.3389/fpls.2024.1324817

Polgári, D., Sepsi, A., Kis, A., **Ali, M**., & Sági, L. (2024) "Intergeneric wheat × barley (*Triticum aestivum* × *Hordeum vulgare*) hybrids: Production, Analysis and Deployment" *Methods in Molecular Biology*, (In press).