Loss of centromeric histone H3 (CENH3) from centromeres precedes uniparental chromosome elimination in interspecific barley hybrids

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AUTHOR SUMMARY

Chromosome elimination, the removal of one parental genome after an egg is fertilized by sperm from another species, is one consequence of an interspecific hybridization event. This process results in the formation of haploid embryos, which have half the normal number of chromosomes. Chromosome elimination has been exploited in barley and other species (e.g., wheat, potato) to produce doubled haploids for breeding and mapping purposes. Doubled haploids allow breeders to produce genetically homoygous plants in a single generation, whereas breeding techniques, such as pedigree or backcrossing, require several generations to obtain high levels of homoygosity. Although this process was initially described 40 y ago by Kasha and Kao (1), the actual cellular mechanism involved in the process of uniparental chromosome elimination remains poorly understood.

Here, we describe the mechanism underlying the selective elimination of paternal chromosomes during the early development of Hordeum vulgare × Hordeum bulbosum (common barley × bulbous barley) hybrid embryos. We found that uniparental chromosome elimination in interspecific barley hybrids is preceded by the loss of a histone H3 variant known as the centromere-specific histone H3 variant (CENH3) from centromeres, regions of chromatin where chromosomes are attached to the spindle and which are required for chromosome segregation. In stable species combinations, despite the presence of different CENH3 transcripts, not all parental CENH3 variants are incorporated into centromeres.

To produce haploids of cultivated barley, crosses are made with H. bulbosum, a close relative of cultivated barley. Chromosomes of H. bulbosum are eliminated several days after pollination (2), depending on the genotype and environmental conditions. To test whether the parent-specific inactivation of centromeres is involved in the mitosis-dependent process of chromosome elimination in interspecific hybrids, we analyzed CENH3 in chromosomally unstable and stable H. vulgare × H. bulbosum combinations. CENH3 was selected for our study because it is located on the chromosome at the assembly site for the kinetochore complex of only active centromeres; moreover, loss of CENH3 interferes with centromere formation and chromosome segregation (3).

We first analyzed the mitotic behavior of unstable hybrid embryos. Chromosomes of H. bulbosum lagged behind H. vulgare chromosomes. In addition, sister chromatids of H. bulbosum segregated asynchronously at anaphase, and consequently formed micronuclei, indicating that attachment of H. bulbosum centromeres to the spindle was defective during cell division. Immunostaining of H. vulgare × H. bulbosum embryos with an antibody that cross-reacts with CENH3s of H. vulgare and H. bulbosum demonstrated that uniparental centromere inactivation is the cause of mitosis-dependent chromosome elimination in wide hybrids. Active centromeres were CENH3-positive, whereas inactive H. bulbosum centromeres were CENH3-negative. Because CENH3 is a stable protein (3), sperm-derived centromere-incorporated CENH3 proteins are likely to provide residual kinetochore function of H. bulbosum until centromeric CENH3 of H. bulbosum falls below the critical level required for correct chromosome segregation, which then results in chromosome elimination. Because silencing or biased ex-
pression of homologous genes has been well documented in both natural and synthetic hybrids, we next tested whether the CENH3 gene of *H. bulbosum* undergoes silencing in unstable hybrids. To do this, we cloned CENH3 genes from both grasses and identified two CENH3 variants in each, known as *HvαCENH3* and *HvβCENH3* in *H. vulgare* and *HbαCENH3* and *HbβCENH3* in *H. bulbosum*. Phylogenetic analysis showed that *Hordeum* αCENH3s cluster with CENH3s of other grasses.

These results indicate that the αCENH3 type is the evolutionarily older variant and that βCENH3, located on chromosome 6H in both *Hordeum* species, likely originated via the duplication of αCENH3, which is encoded by chromosome 1H.

To test whether *H. bulbosum*-specific CENH3 inactivation occurs, we determined the transcriptional activity of parental CENH3s in unstable and stable hybrid embryos and found that the expression patterns were similar regardless of the embryo age. Thus, all CENH3 variants of both parental genomes undergo transcription before elimination, and uniparental silencing of *HbCENH3* genes is not the cause of chromosome elimination in unstable hybrids. Therefore, we assume that in unstable *H. vulgare × H. bulbosum* hybrids, no incorporation of CENH3 protein into the centromeres of *H. bulbosum* takes place. The regulation of CENH3 loading and assembly into centromeres is mediated by a number of proteins, and the erroneous function of any of these will result in a nonfunctional centromere (reviewed in 4). In unstable hybrids, we noticed a different degree of condensation between both parental chromosomes; *H. bulbosum* chromosome condensation was often delayed. Because the correct time of CENH3 deposition seems essential for normal cell division, cell cycle asynchrony (e.g., because of genotypic differences) might interfere with the loading of *H. bulbosum* nucleosomes with CENH3 in unstable hybrids. This, however, does not exclude the possibility that other factors may result in failure to assemble active *H. bulbosum* centromeres in unstable hybrids. On the basis of these observations, we propose a possible model for the mitosis-dependent process of uniparental chromosome elimination in *H. vulgare × H. bulbosum* hybrid embryos (Fig. 1). After fertilization of the *H. vulgare* egg by the *H. bulbosum* sperm, all parental CENH3 genes are transcriptionally active. Translation of *HvCENH3s* occurs, but whether translation of *HbCENH3s* takes place is not known. *HvCENH3* is then loaded into the centromeres of *H. vulgare* but not into those of *H. bulbosum*. Because of cell cycle asynchrony of the two parental genomes, CENH3 incorporation probably only occurs in the centromeres of *H. vulgare* during interphase after replication of DNA. A low temperature during early embryogenesis will support centromere activity of both parental genomes. In unstable hybrids, *H. bulbosum* chromosomal lag because of centromere inactivity during anaphase, subsequently forming micronuclei. Finally, micronucleated *H. bulbosum* chromatin degrades, and a haploid *H. vulgare* embryo will develop.

Duplication and maintenance of two or more copies of CENH3 seem to be rare events in both plant and animal species. Both variants are transcribed in barley, but it is not clear whether these variants are also functional. To characterize the chromosomal distribution of multiple CENH3 proteins in a diploid organism, immunofluorescence experiments were performed with antibodies specific to either αCENH3 or βCENH3 of *H. vulgare*. Both CENH3 variants were detected in the centromeric chromatin of mitotic and meiotic chromosomes and are apparently equally involved in the formation of active centromeres. To investigate whether both CENH3s of *H. vulgare* are incorporated into centromeres of alien chromosomes, we studied stable combinations of *H. vulgare × H. bulbosum* and *Triticum aestivum × H. vulgare*. Our analysis demonstrates that cross-species incorporation of CENH3s occurs in stable species combinations, because both αCENH3 and βCENH3 of *H. vulgare* were detected in all centromeres of stable interspecific *H. vulgare × H. bulbosum* plants. However, because antibodies specific for CENH3s of *H. bulbosum* were not available, it was not possible to determine whether CENH3s of *H. bulbosum* are incorporated into the centromeres of *H. vulgare × H. bulbosum* hybrids. A different situation was observed for αCENH3 and βCENH3 of *H. vulgare* in wheat-barley 1H + 6H addition lines. Despite transcription of both barley CENH3 variants, only *HvαCENH3*-specific immunosignals were detected. These results suggest that species-specific incorporation of CENH3 does not occur if CENH3 from both parents coexist in stable hybrids. However, not all parental CENH3 variants necessarily undergo centromere incorporation when multiple CENH3s coexist. In future studies, it will be interesting to determine the number and origin of different CENH3 variants that are incorporated into the centromeres of polyploid species. To what extent does the cross-capability between species depend on the ability of centromeres to incorporate different parental CENH3 variants? Does each CENH3 variant use its own set of assembly factors in hybrids?

Temperature-mediated changes in nucleosome composition via altered deposition of histone variants by chaperons have recently been demonstrated for plants (5). If temperature-mediated changes in centromeric nucleosome assembly occur, the temperature effect on the process of uniparental chromosomes could be explained. However, it is not known whether the chaperons involved in CENH3 loading are temperature-sensitive.

To sum up, we report four major conclusions regarding the role of CENH3 in chromosomally stable and unstable inter-specific combinations: (i) Diploid barley species encode two CENH3 variants whose gene products are intermingled throughout mitotic and meiotic centromeres; (ii) in stable species combinations, cross-species incorporation of CENH3 occurs despite centromere-sequence differences, although not all CENH3 variants are incorporated into centromeres when multiple CENH3s are present in species combinations; (iii) centromere inactivity of *H. bulbosum* chromosomes triggers the mitosis-dependent process of uniparental chromosome elimination in unstable *H. vulgare × H. bulbosum* hybrids; and (iv) centromere inactivity results from centromeric loss of CENH3 rather than uniparental silencing of CENH3 genes. As well as providing a better understanding of parental genome interaction in newly formed hybrids, our findings could help establish more efficient methods for generating haploids or additional species combinations.

Plant Growth Conditions and Crossing Approaches. Two genotypes of *Hordeum bulbosum* (Cb2920/4 and Cb3811/3) (1) were vegetatively propagated and vernalized for 7–8 wk at 4 °C, with an 8-h day length. Vegetative propagation is necessary because *H. bulbosum* is self-incompatible (2) and individual genotypes cannot be established from seeds. After vernalization, the two genotypes were maintained separately in cool glasshouses (temperatures <18 °C) with a 16-h day length.

For the *H. vulgare* (“Emir”) plants, two environments were used with contrasting temperatures to control chromosome elimination after pollination. One glasshouse was maintained with temperatures greater than 18 °C for chromosome elimination, whereas the other had temperatures less than 18 °C to promote retention of the parental chromosomes after pollination with *H. bulbosum*. Plants were cultivated until ear emergence in a cool glasshouse and were then transferred to their respective environments.

Crossing was done conventionally by emasculating florets of the female parent before anthesis; the spikes covered with bags to prevent out-pollination and pollinated with freshly collected pollen from the male parent. A fine spray of plant growth regulators was applied to florets 1 d (summer) or 1 and 2 d (winter) after pollination to stimulate seed development and improve the quality of the seeds. The mixture comprised 75 mg/L gibberellic acid plus 1 mg/L dicamba, with or without 2 mg/L 2,4-dichlorophenoxyacetic acid. Twelve drops per liter of Tween 20 was added as a surfactant. Immature embryos of various sizes were excised under a stereomicroscope for further analysis.


![Fig. S1.](image1.png) Confirmed anti-grass CENH3 cross-reactivity with CENH3s of *H. bulbosum* by indirect immunostaining. Immunostaining of metaphase (A) and interphase (B) nuclei of *H. bulbosum* with anti-grass CENH3. (Scale bar: 10 μm.)

![Fig. S2.](image2.png) *H. vulgare* × *H. bulbosum* hybrid anaphase cells show lagging chromosomes (arrowheads). (A), (B), and (C) show different examples. Immunostaining with anti-grass CENH3 and anti-α-tubulin. (Scale bar: 10 μm.)
Fig. S3. Comparison of αCENH3 and βCENH3 of *H. vulgare* and of *H. bulbosum* with CENH3s of maize, rice, and sugar cane. Hb, *H. bulbosum*; Hv, *H. vulgare*; Os, rice; So, sugar cane; Zm, maize. (A) Alignment of deduced amino acid sequences. The CENH3-typical α1-helix, α2-helix, α3-helix, loop 1 region, and CAT domain are indicated. (B) Phylogenetic analysis shows that the αCENH3s form a distinct subcluster with the CENH3s of maize, rice, and sugar cane. βCENH3s of *Hordeum* species form a separate cluster. CENH3 of *A. thaliana* was used as an outgroup. At, *A. thaliana*.
Fig. S5. Transcriptional analysis of αCENH3 (A) and βCENH3 (B) of H. vulgare and H. bulbosum in stable and unstable plants 5 and 7 d after pollination (DAP) of old H. vulgare × H. bulbosum hybrid embryos by RT-PCR. Only H. bulbosum-derived αCENH3 and βCENH3 transcripts were digestible with AlwI and BamHI, respectively. As a control, RT-PCR products of H. vulgare and H. bulbosum plants were used. DAP, days after pollination; H.b., H. bulbosum; H.v., H. vulgare.

Fig. S4. Chromosome mapping of αCENH3s and βCENH3s in H. vulgare (A and B) and in H. bulbosum (C and D). Mapping of αCENH3s (A) and βCENH3s (B) of H. vulgare using wheat-barley addition lines. The bands corresponding to the barley CENH3s are indicated by arrowheads. PCR was performed on the genomic DNA samples of (from left to right) wheat (Chinese Spring), barley (Betzes), and seven barley (Betzes) chromosome addition lines in wheat (Chinese Spring) (1, 2). Because the 1H addition line causes severe sterility, double-disomic addition 1H and 6H was used. For amplification of HvαCENH3 and HvβCENH3, the primer pairs 8/9 and 3/10 were used, respectively. Chromosome mapping of αCENH3s and βCENH3s of H. bulbosum using H. vulgare lines with substituted H. bulbosum chromosomes. (C) Amplification of αCENH3 in H. bulbosum but not in the substitution lines (2H–7H) (3, 4), except for 1H, indirectly indicates that αCENH3 is located on 1H of H. bulbosum. A substitution with 1H of H. bulbosum is not available. (D) Amplification of the chromosomal location of HbβCENH3 using the 6H H. bulbosum substitution line in the barley genome; the result shows that this gene is located on chromosome 6 of H. bulbosum. For amplification of HbαCENH3 and HbβCENH3, the primer pairs 4/5 and 3/11 were used, respectively. Amplification of GAPDH with primers 12 and 13 was used as a positive control.

1. Islam AKMR, Shepherd KW (2000) Isolation of a fertile wheat-barley addition line carrying the entire barley chromosome 1H. Euphytica 111:145–149.
Fig. S6. Distribution of αCENH3 and βCENH3 at different stages of mitosis (A) and meiosis (B) in *H. vulgare* is demonstrated by immunostaining. (Scale bars: 10 μm.)
Fig. S7. Confirmation of anti-HvαCENH3 and anti-HvβCENH3 species specificity by indirect immunostaining of H. bulbosum and T. aestivum chromosomes. Anti-grass CENH3 was used as a positive control. (Scale bar: 10 μm.)
Fig. S8. Cross-species incorporation analysis of αCENH3 and βCENH3 of *H. vulgare* in a closely related species. All centromeres of stable *H. vulgare* × *H. bulbosum* hybrid nuclei (A) and of an *H. vulgare*- *H. bulbosum* 7H substitution incorporating αCENH3 and βCENH3 derived from *H. vulgare* (B) are shown. (C) Genotype confirmation of an *H. vulgare*- *H. bulbosum* 7H substitution line by in situ hybridization. Mitotic *H. vulgare*- *H. bulbosum* 7H substitution chromosomes after F1 generation using genomic *H. bulbosum* DNA (green); (AGGGAG)n, a barley-specific centromere repeat (yellow); and 5S rDNA (red) as probes. Circles

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F, forward; R, reverse.