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

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Uniparental chromosome elimination occurs in several interspecific hybrids of plants. We studied the mechanism underlying selective elimination of the paternal chromosomes during the early development of *Hordeum vulgare* × *Hordeum bulbosum* embryos. The following conclusions regarding the role of the centromere-specific histone H3 variant (CENH3) in the process of chromosome elimination were drawn: (*i*) centromere inactivity of *H. bulbosum* chromosomes triggers the mitosis-dependent process of uniparental chromosome elimination in unstable *H. vulgare* × *H. bulbosum* hybrids; (*ii*) centromeric loss of CENH3 protein rather than uniparental silencing of *CENH3* genes causes centromere inactivity; (*iii*) in stable species combinations, cross-species incorporation of CENH3 occurs despite centromere-sequence differences, and not all CENH3 variants get incorporated into centromeres if multiple CENH3s are present in species combinations; and (*iv*) diploid barley species encode two *CENH3* variants, the proteins of which are intermingled within centromeres throughout mitosis and meiosis.

kinetochore | interspecies hybridization | micronuclei | wide crosses

Chromosome elimination of one parental genome after fertilization of the egg by the sperm of another species is a fairly common phenomenon and results in the formation of haploid embryos. It has been exploited for barley (1) and other species (e.g., wheat, potato) to produce doubled haploids for breeding and mapping purposes (reviewed in 2). The advantage of doubled haploids for breeders is that homozygosity can be achieved in the first generation, whereas in breeding systems, such as pedigree or backcrossing, several selfed generations are needed to obtain high levels of homozygosity.

To produce haploids of barley, crosses are made with *Hordeum bulbosum Linnaeus* (bulbous barley grass), a close relative of cultivated barley in the secondary gene pool. Chromosomes of *H. bulbosum* are eliminated several days after pollination (1, 3–6) independent of the crossing direction (1), but hybrids combining both sets of parental chromosomes can be obtained (7). Chromosome elimination is known to depend on genetic factors (7) and temperature after fertilization (8).

Several explanations have been proposed to account for uniparental chromosome elimination [e.g., difference in timing of essential mitotic processes attributable to asynchronous cell cycling (9), asynchrony in nucleoprotein synthesis leading to a loss of the most retarded chromosomes (3, 10)]. Other hypotheses that have been put forward are the formation of multipolar spindles (5), spatial separation of genomes during interphase (11, 12), and genome elimination by nuclear extrusions (4, 13). In addition, degradation of alien chromosomes by host-specific nucleases (14), uniparental nondisjunction of anaphase chromosomes (15), and parent-specific inactivation of centromeres (11, 16–19) have been suggested. The actual cellular mechanism involved in the process of uniparental chromosome elimination remains poorly understood.

To test whether parent-specific inactivation of centromeres is involved in the mitosis-dependent process of chromosome elimination in interpecific hybrids, we analyzed the centromere-specific histone H3 variant (CENH3) [originally called CENP-A in humans (20) and HTR12 in *Arabidopsis thaliana* (21)] in chromosomally unstable and stable *Hordeum vulgare* × *H. bulbosum* combinations. CENH3 was selected for our study because in mammals (22), *Caenorhabditis elegans* (23), and *Drosophila melanogaster* (24), its loss results in the failure of centromere formation and chromosome segregation. A region in CENH3 defined as the centromere targeting domain (CATD) is critical for centromeric localization of CENH3 in various species (25–27). The CATD is composed of the loop1 linker and α2-helix of CENH3 (28, 29), and its substitution enabled the incorporation of an H3 chimera into centromeres (26). This domain mediates molecular recognition events before and after nucleosome assembly and is important for binding of CENH3 to centromeric DNA (27, 28, 30), to CENH3-specific chaperones (31–33), and to CENH3-stabilizing factors (34, 35).

Although centromeric DNA sequences are extremely diverse, all eukaryotic centromeres contain CENH3 (36). The chromosomal location of CENH3 is the assembly site for the kinetochore complex of active centromeres. Any error in histone gene transcription, translation, modification, or import could affect the ability to assemble intact CENH3 chromatin, which would result in the loss of CENH3 from centromeres and, hence, of centromere identity (reviewed in 37). In contrast to conventional histones, CENH3 is rapidly evolving and shows signatures of adaptive evolution in some species (38). ChIP data indicated that CENH3 interacts with *H. vulgare* with *CEREBA*, a centromeric retroelement-like element conserved among cereal centromeres, and with *H. bulbosum*- and *H. vulgare*-specific GC-rich centromeric satellite sequences (39).

The present work provides insights into the role of CENH3 in the process of selective elimination of paternal chromosomes during the development of *H. vulgare* × *H. bulbosum* hybrid embryos. In addition, we identified two functional CENH3s in both diploid barley species and assayed their incorporation into centromeres of alien chromosomes. We found that despite the presence of transcripts, not all parental CENH3 variants are incorporated into centromeres if multiple CENH3s coexist in

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See Commentary on page 13361.

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species combinations. Thus, the lack of cross-species CENH3 incorporation might act as a barrier to species hybridization.

Results

Uniparental Elimination of Chromosomes in Unstable *H. vulgare* × *H. bulbosum* Hybrids Is Accompanied by Loss of CENH3 from Centromeres. The fertilization of the *H. vulgare* egg by the *H. bulbosum* sperm is followed by complete elimination of the *H. bulbosum* genome. Depending on the genotype and on environmental conditions, most of the *H. bulbosum* chromatin is eliminated after pollination in almost all embryos within 1 wk (2). We first analyzed the mitotic behavior of *H. bulbosum* chromosomes in dividing cells of unstable hybrid embryos derived from *H. vulgare* × *H. bulbosum* (Cb2920/4). To promote chromosome elimination, a temperature above 18 °C was used for cultivation of pollinated *H. vulgare* plants. In addition to *H. bulbosum* chromosomes with normal mitotic movements (Fig. 1*A*), between 20% and 70% of anaphase cells showed abnormally segregating *H. bulbosum* chromosomes (Fig. 1*B*). Such chromosomes of *H. bulbosum* lagged behind *H. vulgare* chromosomes, and the sister chromatids segregated asymmetrically at anaphase. As previously described (4), the level of mitotic chromosome condensation partly differed between the parental genomes; chromosomes of *H. bulbosum* were often less condensed. These observations are consistent with a loss of paternal chromosomes during cell division via lagging chromosomes that later form micronuclei (3).

The centromere activity of hybrid cells undergoing uniparental chromosome elimination was determined via immunostaining with grass CENH3- and α-tubulin–specific antibodies. Before that, the cross-reactivity of the anti-grass CENH3 antibody used (40) with CENH3 of *H. bulbosum* was confirmed (Fig. S1 *A* and *B*). Fig. 2*A* shows typical results obtained in anaphase cells in 3- to 6-d-old unstable hybrid embryos. CENH3-positive active centromeres were found in segregating chromatids, whereas lagging chromosomes were depleted of CENH3 (Fig. 2*A* and Fig. S2 *A*–*C*). Neither a primary constriction nor a clear interaction between α-tubulin fibers and kinetochores of lagging *H. bulbosum* chromosomes was recognizable. As a control, all centromeres of stable *H. vulgare* × *H. bulbosum* (Cb3811/3) hybrids cultivated below 18 °C displayed CENH3 signals (Fig. 2*B*).

In addition, embryonic nuclei of unstable hybrids displayed two classes of CENH3 signals. Approximately seven discrete CENH3 signals of strong intensity, together with CENH3 signals of less intensity, per nucleus were found (Fig. 3 *B* and *E*). Subsequent FISH with differentially labeled genomic DNA of *H. bulbosum* (Fig. 3*C*) and with the *Hordeum* centromere-specific probe BAC 7 (41) (Fig. 3*D*) confirmed the hybrid nature of those nuclei. Only approximately 7 of the 14 more or less equally sized

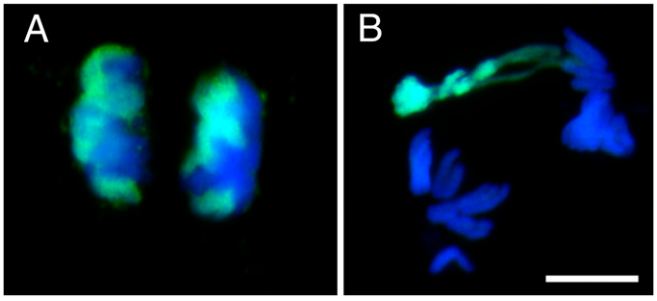

Fig. 1. Anaphase chromosome segregation behavior of normally segregating (*A*) and lagging (*B*) *H. bulbosum* chromosomes in an unstable *H. vulgare* × *H. bulbosum* hybrid embryo. Chromosomes of *H. bulbosum* (green) were identified by genomic in situ hybridization using labeled genomic DNA of *H. bulbosum*. Chromosomes of *H. vulgare* are shown in blue. Note the lagging chromosomes of *H. bulbosum* in *B*. (Scale bar: 10 μm.)

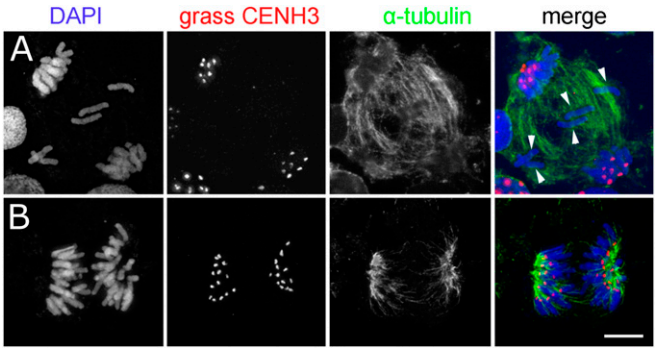

Fig. 2. Anaphase chromosomes of an unstable (*A*) and stable (*B*) *H. vulgare* × *H. bulbosum* hybrid embryo after immunostaining with anti-grass CENH3 and anti–α-tubulin. The centromeres of lagging chromosomes (arrowheads) are CENH3-negative. (Scale bar: 10 μm.)

centromeric FISH signal clusters overlapped with the position of strong CENH3 signals (Fig. 3*E*), suggesting that interphase centromeres of *H. bulbosum* also carry less CENH3 protein. Thus, centromere inactivity of *H. bulbosum* chromosomes, by means of a loss of CENH3, triggers the mitosis-dependent process of uniparental chromosome elimination in unstable *H. vulgare* × *H. bulbosum* hybrids.

At the end of mitosis during reformation of nuclear membranes, lagging chromosomes form micronuclei (42, 43). To determine the centromeric and transcriptional activity of micronucleated *H. bulbosum* chromatin (Fig. 4*A*), the presence of CENH3 and of the RNA polymerase II was assayed. Most micronuclei were CENH3-negative (Fig. 4*B*), and only occasionally were weak CENH3 immunosignals seen. RNA polymerase II signals were distributed homogeneously throughout the nucleoplasm of normal nuclei (Fig. 4*C*), whereas micronuclei were weakly immunolabeled (Fig. 4*C*). Because heterochromatinization of eliminated chromatin is a well-known phenomenon in many nonplant organisms (44), we tested next whether micronuclei are enriched in the heterochromatin marker histone H3 dimethylated at lysine 9 (H3K9me2). Around 60% of 233 micronuclei analyzed revealed an enhanced level of H3K9me2 compared with normal nuclei (Fig. 4*D*). Hence, the transcriptional activity of micronucleated chromatin is strongly reduced. Because the centromeres of micronucleated chromosomes are inactive, no further segregation of *H. bulbosum* chromosomes is possible after micronucleus formation.

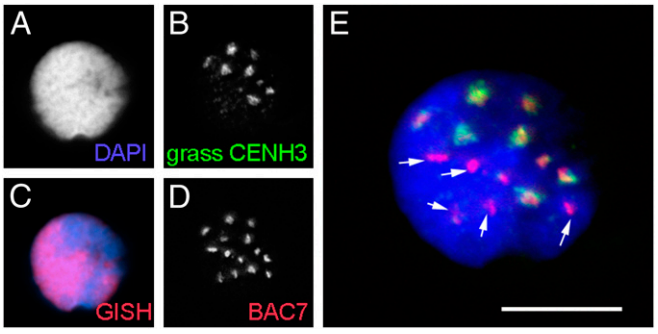

Fig. 3. Interphase nucleus of an unstable *H. vulgare* × *H. bulbosum* hybrid embryo (*A*) after immunostaining with anti-grass CENH3 (*B*), genomic in situ hybridization with *H. bulbosum* DNA (*C*, red), and in situ hybridization with the *Hordeum* centromere-specific probe BAC7 (*D*). GISH, genomic in situ hybridization. (*E*) Only approximately 7 of the 14 more or less equally sized centromeric FISH signal clusters are overlapping with the position of strong CENH3 signals. Hence, interphase centromeres of *H. bulbosum* carry less CENH3 protein. BAC7-positive centromeres without CENH3-signals are shown (arrows). (Scale bar: 10 μm.)

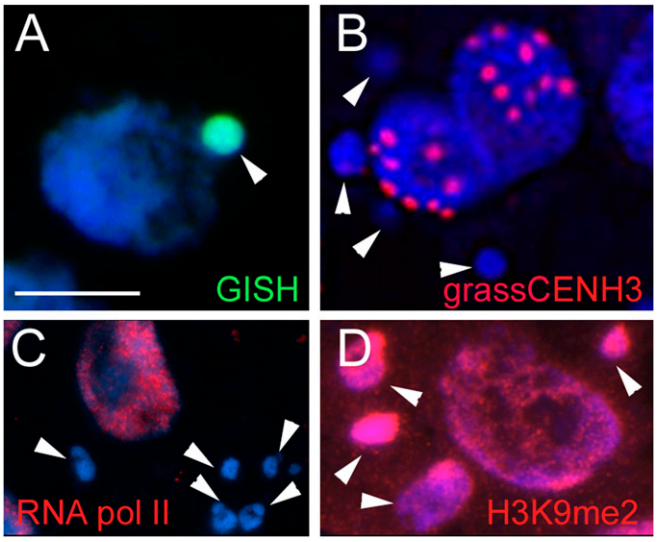

Fig. 4. Characterization of micronuclei of unstable *H. vulgare* × *H. bulbosum* hybrid embryos. Micronuclei are *H. bulbosum*-positive after genomic in situ hybridization (*A*) CENH3-negative (*B*) and RNA polymerase II-negative (*C*) after immunostaining but enriched in H3K9me2-specific heterochromatin-specific markers (*D*). Arrowheads indicate micronuclei. (Scale bar: 10 μm.)

Both *Hordeum* Species Encode Two Active Variants of CENH3. Because silencing or biased expression of homologous genes has been well documented in both natural and synthetic hybrids (45), we first isolated the corresponding genes with the aim of testing whether the CENH3 gene of *H. bulbosum* undergoes silencing in unstable hybrids.

Initial RT-PCR using a monocotyledon-specific *CENH3* primer pair (46) (Table S1, primer pair 1/2) for isolating on *Hordeum CENH3* amplified parts of the two *CENH3* variants in *H. vulgare* called *Hv*α*CENH3* and *Hv*β*CENH3*. BLAST analysis against the National Center for Biotechnology Information database resulted in the identification of the 5′ region of *Hv*α*CENH3* (GenBank accession no. BU996921). The coding sequence of *Hv*β*CENH3* (GenBank accession no. AK249602) was completed by 3′ RACE PCR using primer 3 (Table S1). Based on the *H. vulgare CENH3* sequences, primers were designed to amplify both *CENH3*s of *H. bulbosum*, named *Hb*α*CENH3* and *Hb*β*CENH3* (Table S1, primer pairs 14/15 and 1/2 for *Hb*α*CENH3* and primer pair 6/7 and primer 3 for *Hb*β*CENH3* were used for RACE PCR). The deduced amino acid sequences of the identified CENH3s [GenBank accession nos. JF419328 (*Hv*α*CENH3*), JF419329 (*Hv*β*CENH3*), GU245882.1 (*Hb*α*CENH3*), and JF419330 (*Hb*β*CENH3*)] were compared with CENH3 of maize (47), rice (40), and sugar cane (48) to determine the conserved α*N*-helix, α1-helix, α2-helix, α3-helix, and CENH3-specific (26) loop1 regions (Fig. S3*A*). Despite the first five completely conserved amino acids, the N-terminal tails were highly variable. βCENH3 types have a shorter N-terminal region than αCENH3 types and a longer loop1 region.

Phylogenetic analysis of the amino acids of *Hordeum* CENH3s with further plant CENH3s (Fig. S3*B*) showed that αCENH3s and βCENH3s of *Hordeum* species form two distinct subclusters. In addition, *Hordeum* αCENH3s cluster with the CENH3s of rice, maize, and sugar cane. Therefore, it is likely that α and β types of CENH3 diverged before the speciation of *H. vulgare* and *H. bulbosum*. Chromosome mapping of *CENH3*s confirmed this assumption (Fig. S4). α*CENH3* is encoded by chromosome 1H in *H. vulgare* and in *H. bulbosum*; *CENH3* of rice maps to chromosome 5, which is syntenic to barley 1H (49). Furthermore, β*CENH3* is located on chromosome 6H in both *Hordeum* species.

***CENH3s* of Both Parental Genomes Are Transcribed in Chromosomally Stable and Unstable Hybrid Embryos.** To test whether *H. bulbosum*-specific *CENH3* inactivation occurs, the transcriptional activity of parental *CENH3*s in unstable hybrids was determined. Stable and unstable hybrid embryos of different ages (5–7 d after pollination) were isolated, two to five embryos of each age and type were pooled, and RNA was isolated for cDNA synthesis. To detect transcripts, we used α*CENH3* (primer pair 4/5) or β*CENH3* (primer pair 6/7) type-specific primer pairs. *H. vulgare*- and *H. bulbosum*-derived *CENH3* transcripts were discriminated via species-specific restriction sites. AlwI cleavage of the *H. bulbosum*-derived PCR products generates two fragments of 210 bp and 54 bp, leaving *H. vulgare*-derived α*CENH3* fragments undigested (Fig. S5*A*). Similarly, BanII cleavage of *H. bulbosum*-derived β*CENH3* amplicons results in fragments of 398 bp and 234 bp, whereas *H. vulgare*-derived transcripts are unaffected (Fig. S5*B*). BanII and AlwI cleavage of *CENH3* transcripts amplified from stable or unstable *H. vulgare* × *H. bulbosum* hybrid revealed transcription of all four parental *CENH3*s (Fig. S5 *A* and *B*). The expression patterns were similar regardless of the embryo age. Thus, all *CENH3* variants of both parental genomes undergo transcription in unstable hybrids, and uniparental silencing of HbCENH3 genes is not the cause of chromosome elimination in unstable hybrids.

Both CENH3 Variants Intermingle in Mitotic and Meiotic Centromeres of *H. vulgare*. Duplication and maintenance of two or more copies of CENH3 seem to be rare events in both plant and animal species. Examples of diploid plants that possess two CENH3s are *Arabidopsis halleri, Arabidopsis lyrata* (50), and *Luzula nivea* (51). Both variants are transcribed, but it is not clear whether they are also functional. To characterize the chromosomal distribution of multiple CENH3s in a diploid organism, antibodies were generated for each variant of *H. vulgare* CENH3. Western blot analysis using in vitro translated HvαCENH3 and HvβCENH3 confirmed the specificity of both antibodies (Fig. 5) because each type of antibody recognized only the corresponding CENH3 variant, whereas anti-grass CENH3 reacted with both types of CENH3.

To determine the chromosomal distribution of αCENH3 and βCENH3 during mitosis and meiosis, immunofluorescence ex-

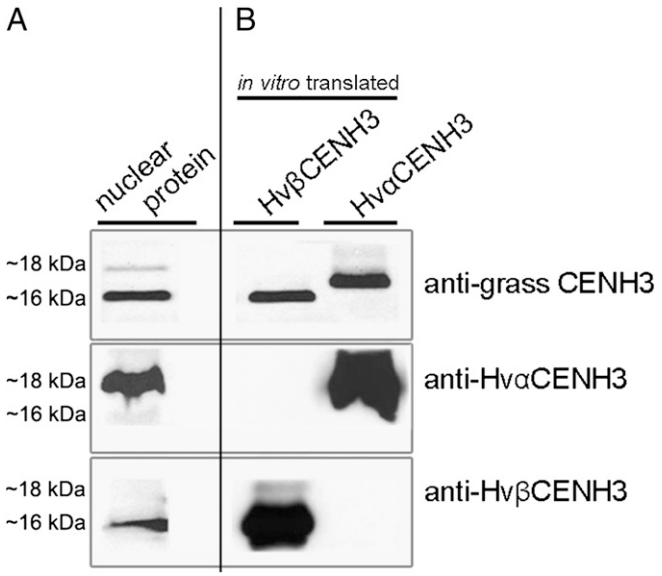

Fig. 5. Western blot analysis demonstrating the specificity of anti-HvαCENH3-, anti-HvβCENH3-, and anti-grass CENH3-specific antibodies on nuclear (*A*) and in vitro translated HvαCENH3 and HvβCENH3 proteins (*B*).

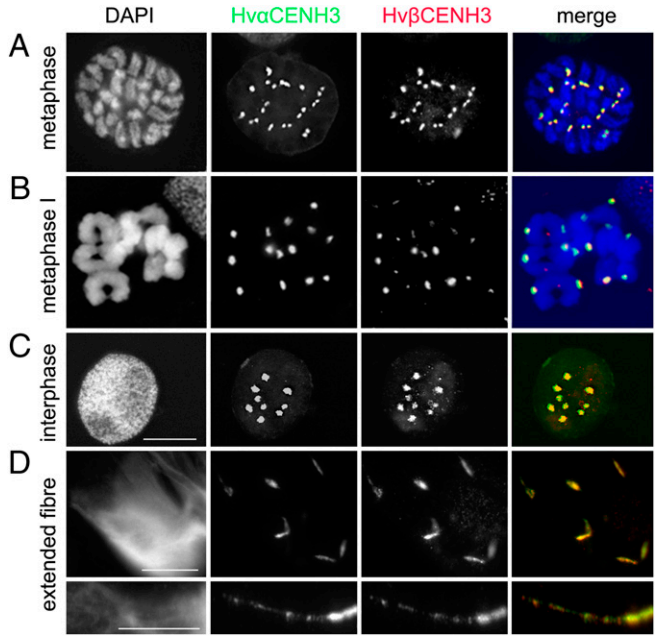

Fig. 6. Distribution of αCENH3 and βCENH3 in mitotic (*A*) and meiotic (*B*) metaphase chromosomes and interphase nuclei of *H. vulgare* (*C*) as demonstrated by immunostaining. An overlap of αCENH3 and βCENH3 signals was found for centromeres as well as for (*D*) up to 12-fold artificially extended centromeres. (Scale bars: 10 μm.)

periments were done. Double immunostaining revealed that αCENH3 and βCENH3 are present in the centromeres of all barley chromosomes at all mitotic stages analyzed (Fig. 6*A* and Fig. S6*A*). Signals of both CENH3 variants always overlapped with the position of the primary constriction. A similar distribution of both CENH3 variants was also detected at different stages of the first and second meiotic divisions (Fig. 6*B* and Fig. S6*B*). The amount of αCENH3 and βCENH3 proteins seems to be comparable, because the intensity of corresponding immunofluorescence signals was generally of similar intensity.

To decipher the organization of αCENH3- and βCENH3-containing centromeric chromatin at higher resolution, immunostaining experiments were done on extended chromatin fibers. For this purpose, chromatin fibers prepared from isolated nuclei of barley leaves were immunolabeled with both CENH3 antibodies. An overlap of αCENH3- and βCENH3-specific signals was observed on centromeres (Fig. 6*C*) but also on up to 12-fold artificially extended centromeres (Fig. 6*D*). We conclude that centromeres can incorporate αCENH3 and βCENH3 in all chromosomes at mitotic and meiotic stages. Both CENH3 variants are intermingled in centromeric chromatin and are apparently equally involved in the formation of barley centromeres.

Although Transcribed, Not All CENH3 Variants Are Deposited on Centromeres if Multiple CENH3s Coexist in Species Combinations. To investigate whether both CENH3s of *H. vulgare* are incorporated into centromeres of alien chromosomes, we studied stable *H. vulgare* × *H. bulbosum* and *Triticum aestivum* × *H. vulgare* combinations. First, we confirmed the species specificity of the barley CENH3 antibodies. Both antibodies did not cross-react with *H. bulbosum* or *T. aestivum* CENH3, whereas anti-grass CENH3 recognized the corresponding protein in all species tested (Fig. S7). Next, double-immunostaining experiments were performed on root nuclei of a stable *H. vulgare* × *H. bulbosum* hybrid plant with anti-grass CENH3 (as a positive control) combined with either HvαCENH3- or HvβCENH3-specific antibodies. Up to14 HvαCENH3 and HvβCENH3 signals overlapped with the position of anti-grass CENH3 signals (Fig. S8*A*). Hence, both CENH3 variants of *H. vulgare* incorporate equally well into the centromeric nucleosomes of *H. bulbosum*.

Next, a stable *H. vulgare-H. bulbosum* 7H substitution line was studied. Chromosome 7H of *H. bulbosum* does not encode any *CENH3* gene. The genotype of the *H. vulgare-H. bulbosum* 7H substitution line was confirmed by in situ hybridization (Fig. S8*C*). After immunostaining, for each CENH3 antibody combination, up to 14 overlapping discrete signals per nucleus were detected (Fig. S8*B*). Hence, α*CENH3* and β*CENH3* of *H. vulgare* can functionally compensate for the missing *CENH3* of *H. bulbosum*.

We then posed the question of whether barley centromeres still specifically incorporate HvCENH3 when barley chromosomes carrying *CENH3* genes are added to the genome of *T. aestivum*, a species less closely related to *H. vulgare* than *H. bulbosum.* When hybrids are formed, the centromere-specific histones of each parental species may operate in the context of a dual set of centromeric sequences. We used a wheat-barley double-disomic 1H + 6H addition line. RT-PCR demonstrated (α*CENH3*: primer pair 8/9, β*CENH3*: primer pair 3/10) the presence of both *H. vulgare CENH3* transcripts in a *T. aestivum* background (Fig. S8*D*). Finally, double immunostaining on nuclei of the characterized wheat-barley addition lines with grass CENH3 (as a positive control), and HvαCENH3- or HvβCENH3-specific antibodies was performed. Anti-HvαCENH3 and anti-grass CENH3 each detected up to 46 overlapping signals per nucleus (Fig. S8*E*), but no centromeric incorporation of HvβCENH3 was found in nuclei of wheat-barley 1H + 6H plants. Thus, no species-specific incorporation of CENH3 occurs if CENH3 of both parents coexists in stable hybrids. However, not all parental CENH3 variants necessarily undergo centromere incorporation if multiple CENH3s coexist.

Discussion

Diploid *Hordeum* Species Encode Two Functional *CENH3* Variants. This report describes two functional *CENH3* variants in diploid grasses. Because two *CENH3* variants exist in *H. vulgare* and *H. bulbosum* genes, duplication of αCENH3 must have occurred at least 7 million years ago, the time when *H. vulgare* and *H. bulbosum* diverged (52). An alternative explanation is that the second gene variant is the remainder of an earlier whole-genome duplication that occurred 20 million years before the divergence of *Oryza*, *Brachypodium*, and *Hordeum* from a common ancestor that existed ∼41–47 million years ago (53). Because *Oryza sativa* and *Brachyopodium distachyon* encode only one *CENH3* variant each, it is possible that the second *CENH3* variant in these grasses was lost. Loss of a second *CENH3* gene copy also occurred in *Zea mays* (47). In maize, the allotetraploidization event probably occurred ∼11.4 million years ago (54). On the other hand, in other allopolyploid organisms, such as *Arabidopsis suecica* (21), wild rice (55), or *Nicotiana tabacum* (56), multiple *CENH3* types were identified.

Despite considerable protein sequence differences in the CATD region, both CENH3 variants of *H. vulgare* are detectable in the centromeric nucleosomes of all mitotic, meiotic, and extended interphase chromosomes of barley. This observation suggests at least two possibilities regarding the centromeric localization of two CENH3 variants. Either both CENH3 variants are incorporated into the same nucleosomes forming heterodimers or barley centromeres are composed of alternate blocks of αCENH3- and βCENH3-containing nucleosomes. The centromeric composition of alternating blocks of CENH3- and H3-containing nucleosomes has previously been demonstrated for metazoan organisms (36, 57) and plants (58). However, because of the limited resolution of light microscopy, our immunostaining experiment on extended chromatin fibers does not exclude the possible formation of αCENH3- and βCENH3-containing heterodimers.

CENH3 Behavior in Stable Species Combinations. 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. Because CENH3s of wheat are probably more similar in sequence to αCENH3 of *H. vulgare*, HvαCENH3 was preferentially incorporated into the centromeres of wheat and barley.

After sexual hybridization, the CENH3 of one parent can promote centromere functionality of the second parent despite centromere sequence differences. However, the CENH3 sequence dissimilarity must not be too severe. This finding is strongly supported by the availability of a large number of chromosome addition lines. Even remotely related species, such as oat and maize, can be sexually hybridized to produce fertile partial hybrids (59). In this species combination, CENH3 of oat compensates for the missing CENH3 of maize, because the maize *CENH3* gene is silenced in the genetic background of oat in oat-maize chromosome addition lines (16). Similarly, the CENH3 protein from *A. thaliana* can be detected in the centromeres of all chromosomes of allotetraploid *A. thaliana* × *Arabidopsis arenosa* hybrid *A. suecica* (21). Our observation based on sexually generated hybrids supports previous cross-species CENH3 incorporation experiments in transgenic organisms (27, 51, 60–62), for which it was reported that CENH3s of closely related species can target centromeres in alien species.

It will be interesting to study how many different CENH3 variants are incorporated into centromeres of polyploid species. To what degree 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? However, in a transgenic situation, CENH3 of a closely related species could get incorporated alone (62) or in combination with WT CENH3 (51, 60) with help from the loading machinery of the host organism. Transgenic complementation of an inactive version of *CENH3* was only possible if the heterologous *CENH3* originated from a closely related species (62). For instance GFP-CENH3 from the close relative *A. arenosa* complemented *A. thaliana cenh3-1*, which is consistent with the observation that *A. thaliana* CENH3 localizes to both *A. thaliana* and *A. arenosa* chromosomes in the allopolyploid species *A. suecica* (21). However, GFP-CENH3 from a closely related *Brassica* species was targeted to centromeres but did not complement *cenh3-1*, indicating that kinetochore localization and centromere function of alien CENH3 can be uncoupled (62).

Centromeric Loss of CENH3 Protein Is Involved in the Process of Uniparental Chromosome Elimination. CENH3 immunostaining of *H. vulgare* × *H. bulbosum* embryos demonstrated that uniparental centromere inactivation is, as previously postulated (11, 16–19), a cause of mitosis-dependent chromosome elimination in wide hybrids. Active centromeres of *H. vulgare* and *H. bulbosum* were CENH3-positive, whereas inactive *H. bulbosum* centromeres were CENH3-negative or the amount of CENH3 was reduced in unstable hybrids.

Elimination *of H. bulbosum* chromosomes is completed by 5–9 d after pollination (3–5). Because CENH3 is a stable protein (22), sperm-derived centromere-incorporated CENH3 proteins are likely to provide a residual kinetochore function of *H. bulbosum* until it falls below a level critical for correct chromosome segregation, resulting in chromosome elimination. That a limited fraction of parental H3 variants is transmitted to the progeny has been demonstrated for animal embryos (22). If preexisting CENH3 is partitioned equally between duplicated sister centromeres and no de novo incorporation of CENH3 into *H. bulbosum* centromeres occurs, its amount will be approximately halved with each cell division. In humans, even 10% of the endogenous CENH3 can aid in efficient kinetochore assembly (63). On the other hand, a reduced CENH3 amount can trigger centromere inactivity in neocentromeres of maize (64). Nevertheless, if a zygotic resetting of CENH3, as demonstrated for GFP-tagged CENH3 in *A. thaliana* (65, 66), also exists in grasses, active removal of both parental CENH3s before the first zygote division would occur and the reactivation of *H. bulbosum* centromeres in unstable hybrids would be diminished.

Despite transcription of all parental *CENH3* genes in unstable *H. vulgare* × *H. bulbosum* hybrids and the existence of cross-species incorporation of CENH3s in stable hybrids, the centromeres of *H. bulbosum* in unstable hybrids had a reduced content of CENH3. Therefore, we assume that in unstable *H. vulgare* × *H. bulbosum* hybrids, no incorporation of CENH3 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 67). For example, inactivation of the metazoan CENH3 interacting partner CENP-H, CENP-I, CENP-K, or CENP-N results in reduced assembly of nascent CENH3 into centromeric chromatin and causes defects in kinetochore assembly and chromosome congression (34, 68). However, with the exception of Mis12 of *A. thaliana* (69), no other protein involved in the establishment and maintenance of CENH3 chromatin has been identified in plants.

In unstable hybrids, we noted a different degree of condensation between both parental chromosomes. The condensation process of *H. bulbosum* chromosomes was often delayed (4). In agreement with our observation, Bennett et al. (3) reported that *H. bulbosum* requires more time to complete the cell cycle than *H. vulgare.* Because the correct time of CENH3 deposition seems to be essential (25), cell cycle asynchrony (e.g., attributable to 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 the failure to assemble active *H. bulbosum* centromeres in unstable hybrids. Notably, in interspecific somatic hybrids of different mammals, predominant loss of one parental genome occurs (reviewed in 70). However, it is not known whether a comparable process of chromosome elimination via loss of CENH3 is evolutionarily conserved and would also occur in animal hybrids.

The elimination process of chromosomes can be influenced by environmental conditions (8). A temperature above 18 °C during the early stages of hybrid embryo growth can promote chromosome elimination. How does temperature influence the process of chromosome elimination? Temperature-mediated changes in nucleosome composition via altered deposition of histone variants by chaperons have recently been demonstrated for plants (71). 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 chaperones involved in CENH3 loading are temperature sensitive.

On the basis of the above-mentioned observations, we propose a possible model of how the mitosis-dependent process of uniparental chromosome elimination works in *H. vulgare* × *H. bulbosum* hybrid embryos (Fig. 7). 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 unknown. HvCENH3 is then loaded into the centromeres of *H. vulgare* but not of *H. bulbosum.* Because of cell cycle asynchrony of the two parental genomes, CENH3 incorporation probably only occurs in the

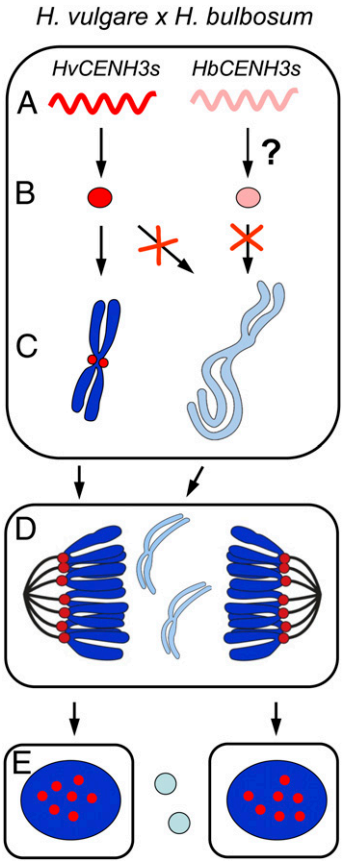

Fig. 7. Proposed model of how the mitosis-dependent process of uniparental chromosome elimination operates in *H. vulgare* × *H. bulbosum* hybrid embryos. (*A*) After fertilization of the *H. vulgare* egg by the *H. bulbosum* sperm, all parental CENH3s are transcriptionally active. (*B*) Translation of HvCENH3s occurs; whether translation of HbCENH3s occurs is not known. (*C*) Loading of CENH3 (red) into the centromeres of *H. vulgare* but not of *H. bulbosum* occurs. As a result of cell cycle asynchrony of the two parental genomes, CENH3 incorporation probably only occurs in the centromeres of *H. vulgare* during G2. A reduced temperature during early embryogenesis promotes normal centromere activity of both parental genomes. (*D*) As a result of centromere inactivity in unstable hybrids, anaphase chromosomes of *H. bulbosum* lag and subsequently form micronuclei. (*E*) Micronucleated *H. bulbosum* chromatin finally degrades, and a haploid *H. vulgare* embryo will develop.

centromeres of *H. vulgare* during G2. A low temperature during early embryogenesis supports centromere activity of both parental genomes. In unstable hybrids, *H. bulbosum* chromosomes lag because of centromere inactivity during anaphase, subsequently forming micronuclei. Finally, micronucleated *H. bulbosum* chromatin will degrade, and a haploid *H. vulgare* embryo will develop. Whether a comparable haploidization process takes place in *A. thaliana cenh3-1* null mutants expressing altered CENH3 proteins that were crossed to WT plants remains to be studied (61).

In summary, we report four major conclusions regarding the role of CENH3 in chromosomally stable and unstable interspecific combinations (1). Diploid barley species encode two CENH3 variants whose gene products are intermingled throughout mitotic and meiotic centromeres (2). In stable species combinations, cross-species incorporation of CENH3 occurs despite centromere-sequence differences. However, not all CENH3 variants get incorporated into centromeres if multiple CENH3s are present in species combinations (3). Centromere inactivity of *H. bulbosum* chromosomes triggers the mitosis-dependent process of uniparental chromosome elimination in unstable *H. vulgare* × *H. bulbosum* hybrids (4). Centromeric loss of CENH3 protein rather than uniparental silencing of *CENH3* genes is causing centromere inactivity.

Materials and Methods

Plant Material, Crossing Procedure, and Preparation of Embryos. A series of crosses were made between diploid *H. vulgare* "Emir" (female parent) and diploid *H. bulbosum* lines Cb2920/4 and Cb3811/3 (pollen donors) (72). For *H. vulgare* plants, two environments were used with different temperatures to control chromosome elimination after pollination. A temperature higher than 18 °C supports chromosome elimination, whereas a temperature lower than 18 °C promotes retention of the parental chromosomes after pollination with *H. bulbosum* (8). Barley spikes were emasculated 1–2 d before anthesis and pollinated 1 d later with freshly collected *H. bulbosum* pollen. Embryo development was stimulated by spraying the spikes with an aqueous solution of gibberellic acid at a rate of 75 ppm 1 d after pollination (details are provided in *SI Materials and Methods*). For subsequent in situ hybridization or indirect immunostaining, 6- to 13-d-old ovaries were isolated and fixed in ethanol/acetic acid (3:1) or 4% (wt/vol) paraformaldehyde in 1× microtubule-stabilizing buffer (MTSB), respectively. Embryos were isolated under a stereomicroscope using fine needles.

Different wheat-barley addition lines as well as barley-*H. bulbosum* substitution lines were used for mapping of CENH3 coding genes (details are provided in Fig. S4).

Isolation of Nuclei and Preparation of Stretched Chromatin Fibers. Nuclei were isolated according to the method of Galbraith et al. (73). Extended chromatin fibers were prepared as described (39). The immunostaining procedure on the stretched chromatin fibers or on nuclei was the same as for squashed cells.

FISH and Indirect Immunostaining. FISH and indirect immunostaining were carried out as described by Gernand et al. (4) and Ma et al. (74), respectively. FISH probes were labeled with Atto-590-dUTP or Atto-488-dUTP (Jena Bioscience) by nick translation. The following primary antibodies were used: rabbit anti-RNA polymerase II CDC phospho Ser5 (catalog no. 39233; diluted 1:100; ACTIVE MOTIF), rabbit anti-histone H3K9me2 (catalog no. 07-441, diluted 1:300; Upstate), mouse anti–α-tubulin (catalog no. T 9026, diluted 1:100; Sigma), and rabbit anti-grass CENH3 (40) (diluted 1:100), as well as HvαCENH3- and HvβCENH3-specific antibodies (diluted 1:100). Epifluorescence signals were recorded with a cooled CCD-camera (ORCA-ER; Hamamatsu). Imaging was performed using an Olympus BX61 microscope and an ORCA-ER CCD camera (Hamamatsu). Deconvolution microscopy was used for superior optical resolution of globular structures. All images were collected in gray scale and pseudocolored with Adobe Photoshop 6 (Adobe). Projections (maximum intensity) were done with the program AnalySIS (Soft Imaging System).

RNA Isolation, RT-PCR, and RACE PCR. RNA was extracted by either the TRIzol method (75) or using the PicoPure RNA Isolation Kit (Arcturus). cDNA was prepared from DNase I-treated RNA using the Reverse Aid H Minus First Strand cDNA Synthesis Kit (Fermantas). PCR included the following steps: 94 °C for 3 min, 40 cycles at 94 °C for 1 min, annealing temperature for 1 min and 10 s, and 72 °C for 1 min and 30 s. Primers and annealing temperatures are given in Table S1. For RACE PCR, the SMART RACE cDNA Amplification Kit (Clontech Company) was used.

Analysis of PCR Fragments and Cleaved Amplified Polymorphic Sequence Analysis. DNA fragments were ligated into the pGEM-T easy vector (Promega) and sequenced using the PGRC Sequencing Service (Leibnitz Institute of Plant Genetics and Crop Plant Research). For cleaved amplified polymorphic sequence analysis, purified PCR products were digested using AlwI or BanII, size-fractionated by gel electrophoresis, and recorded.

Generation of *H. vulgare* CENH3-Specific Antibodies. Suitable CENH3 type-specific epitopes were identified [CQRRQETDGAGTSETPRRAGR and CAEGAPGEPTKRKPHRFR for generating HvαCENH3-specific antibodies (a double-peptide antibody) and CSKSEPQSQPKKKEKRAYR for generating HvβCENH3-specific antibodies]. The corresponding peptides were synthesized and used to immunize guinea pigs for generation of anti-HvαCENH3 antibodies and rabbits for generation of anti-HvβCENH3 antibodies to analyze both CENH3 types simultaneously. Peptide synthesis, immunization of animals, and peptide affinity purification of antisera were performed by Pineda (Antikörper-Service).

In Vitro Protein Synthesis and Western Blot Analysis. For in vitro protein synthesis, the PURExpress cell-free transcription-translation system (New England Biolabs) was used. Quantified protein samples were separated on

10% (wt/vol) SDS/PAGE gels using Tricin-SDS/PAGE running buffers (76), blotted on nitrocellulose membranes, and then incubated first with primary antibodies (diluted 1:1,000) and then with secondary antibodies [anti-rabbit IgG: IRDye800 conjugated, diluted 1:5,000 (LI-COR); anti-guinea pig IgG: HRP-conjugated, diluted 1:5,000 (Dianova)].

Phylogenetic Analysis. Evolutionary analyses was conducted using deduced amino acid sequences of CENH3 in *Z. mays*, *Saccharum officinarum*, *O. sativa, A. thaliana*, and α and β types of *H. vulgare* and *H. bulbosum*. Phylogenetic relationships were estimated by the neighbor joining method (Lasergene).

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