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Analysis of uniparental chromosome elimination in wide crosses

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Abbreviations

2,4-D	2,4-dichlorophenoxy acetic acid
BLAST	basic *l*ocal alignment search tool
bp	base pair
CAPS	cleaved amplified polymorphic sequence
CATD	centromere targeting domain
CCAN	constitutive centromere-associated network
CCS	cereal centromeric sequence
cDNA	complementary deoxyribonucleic acid
CDS	coding *sequence*
CENH3	centromeric histone H3
CENP-A	centromeric protein A
Cereba	centromeric retrotransposons of barley
ChIP-seq	chromatin immunopercipitation-sequencing
Cm	centimeter
CR	centromeric retrotransposon
DAP	days after pollination
DAPI	4',6-diamidino-2-phenylindole
DH	doubled-haploid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminotetra-acetic acid
Fig	figure
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
G1	gap1 phase
G2	gap2 phase
GA3	gibberellic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GISH	genomic *in situ* hybridization
H	hour
H2A	histone H2A
H2B	histone H2B
H3	histone H3
H4	histine H4
HbCENH3	*Hordeum bulbosum* CENH3
HIRA	histone cell cycle regulation defective homolog A
HJURP	holliday junction recognition protein
HmCENH3	*H. marinum* CENH3
HvCENH3	*H. vulgare* CENH3
IgG	Immunoglobulin G
IPTG	iIsopropyl-β-D-thiogalactopyranoside

kb	kilo base
kDa	kilodaltons
KLN-2	kinetochore null mutant
LTRs	long terminal repeats
min	minute
mJ/cm^2	millijoule per square centimeter
ml	milliliters
mM	millimolar
mRNA	messenger ribonucleic acid
MTSB	microtubules stabilizing buffer
MYA	million years ago
NAC	nucleosome associated complex
Ng	nanogram
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
Pg	pictogram
PMSF	phenyl methyl sulfonyl fluoride
RACE	rapid amplification of cDNA ends
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
RSF	remodeling and spacing factor
RT-PCR	reverse transcription polymerase chain reaction
S	synthesis phase
SD	standard deviation
SDS	sodium dodecyl sulfate
SSC	saline-sodium citrate
TaCENH3	*Triticum aestivum* CENH3
TILLING	targeting induced local lesions in genome
UTR	untranslated region
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
α	alpha
β	beta
µg	microgram
µl	microlitre
µm	micrometer
µM	micromole

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Supplementary material

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Appendix Fig. 1. Nucleic acid sequence alignment of *H. vulgare* (HvαCENH3), *H. bulbosum* (HbαCENH3), *H. marinum* (HmαCENH3), *H. vulgare* (HvβCENH3), *H. bulbosum* (HbβCENH3) and of *H. marinum* (Hm βCENH3). Residues that are completely conserved in sequence are shown in white on a black background, those that are ≥80% conserved are shown in white on grey background and ≥60% identical residues are shown in black in grey background. The gaps are shown as dashes. CENH3s are more variable in N-terminal regions and more conserved in C-terminal... 129

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1. Introduction

1.1. Polyploidization

Polyploids are produced by multiplication of the genome derived from a single species (autopolyploid), or combination of two or more divergent genomes from different species (allopolyploid). Polyploidization is a widespread phenomenon in eukaryotes and is predominant in flowering plants (Leitch and Bennett, 1997; Wendel, 2000; Osborn et al. 2003). The formation of allopolyploid requires the adaptation of two nuclear genomes within a common cytoplasm (Lukens et al., 2006).

Interspecific hybridization is common in plants and has played a crucial role in the evolution of plant species by generating new ecotypes or new species and by allowing gene exchanges across species boundaries (Nasrallah et al., 2000). The success of hybridization and gene transfer depends on the level of genetic and structural relatedness between the genomes (Leflon et al., 2006) and the similarity of the centromere structure in the two parents, or expression of relevant function factors of the alien chromosomes in a foreign genetic background (RieraLizarazu et al., 1996). More frequent introgressions are expected when crops and their wild relatives share higher levels of similarity.

70% of plant species appear to have experienced at some point in their history an allopolyploidization event (Masterson, 1994). Several species in the genus *Brassica* (e.g. polyploids: *B. juncea*, *B. napus, B. carinata;* and diploids*: B. rapa, B. nigra,* and *B. oleracea*) (Xu et al., 2009), *Arabidopsis suecica* (a tetraploid derived from the genomes of *A. arenosa* and *A. thaliana*) (Pontes et al., 2004), and several species in the *Triticum* and *Aegilop*s groups have been studied in detail using resynthesized polyploids (Shaked et al., 2001). In natural polyploids, many genes from the two parental genomes contribute unequally to overall gene expression, some showed uniparental expression, whereas others exhibited biased gene expression or additive expression from the two homoeologous

copies. Gene expression biases and silencing also were found to be tissue-specific (Pikaard, 2000; Riddle and Birchler, 2003).

In some complexes, polyploidization is accompanied by drastic genome reorganizations immediately following the actual hybridization event (Riddle and Birchler, 2003). This reorganization includes genetic changes such as the loss or gain of Restriction Fragment Length Polymorphisms (RFLPs), elimination of low-copy sequences or repeated sequences, the activation of transposable elements and gene conversion (Han et al., 2005; Liu et al., 1998; Pontes et al., 2004). In addition, a variety of epigenetic alterations are observed commonly. Epigenetic changes, especially altered cytosine methylation patterns, are assumed to be responsible for altered gene expression states and for the reactivation of transposable elements (Kashkush et al., 2002; Riddle and Birchler, 2003). In some cases, rapid elimination of chromosomes of one of the parental genome after interspecific hybridization have been observed (Campbell et al., 2000; Gernand et al., 2006; Gernand et al., 2005; Moav, 1961).

1.2. Chromosome elimination

Several hybrids are karyotypically unstable. Parent-specific chromosomes are eliminated partially (Fujiwara et al., 1997) or completely (Komeda et al., 2007; Thomas and Pickering, 1983) from the hybrid nuclei. This phenomenon is called as “uniparental chromosome elimination” and has been observed in several interspecific hybrids. Complete chromosome elimination results in the production of haploid genotypes. In the case of incomplete chromosome elimination, several chromosomes of the donor parent can be still found in mature plants. Partial chromosome elimination has facilitated the production of addition lines and gene transfer by inducing intergenomic translocations (Komeda et al., 2007). For example, crossing of *Brassica napus* and *Lesquerella fendleri* has been used successfully for introgression of some interesting genes effective for oil quality (Du et al., 2008). Other examples are the combinations oat × maize and wheat × barley (RieraLizarazu et al., 1996; Thomas and Pickering, 1983).

Somatic combination of remote species via protoplast fusion could be used for transferring genes between plants that are sexually incompatible (Gupta et al., 1984). In somatic genome combinations a positive correlation between the frequency of hybrids with eliminated chromosomes and the genetic distance between the species in each combination was found. Furthermore, by combining species with different ploidy levels they found a significantly higher degree of chromosome elimination compared to combinations of species with the same ploidy level (Sundberg and Glimelius, 1991). In general the combination of parental species determines the degree of chromosome elimination (Komeda et al., 2007).

Chromosome elimination is also a widespread developmental phenomenon for sex determination in insects like "sciarid flies". In this fly chromosome elimination takes place during early embryo development and at the first male meiotic division (Goday and Esteban, 2001). Chromosome elimination is further observed in several other groups of animals, like nematodes and crustaceans. Even in vertebrates like in hagfishes and in frogs of the genus *Rana* chromosome elimination has been reported (Adamowski et al., 1998).

1.3. Application of chromosome elimination for the generation of doubled - haploids for plant breeding

Chromosome elimination in interspecific hybrids is a powerful tool in breeding programs. Total elimination of one genome permits the formation of haploids (Adamowski et al., 1998) and can be used to produce doubled-haploid plants (Houben et al., 2011). Table 1 shows a list of reported examples of wide crosses which resulted in haploids.

This process enables large numbers of doubled-haploid (DH) plants to be obtained for breeding and mapping (Devaux and Pickering, 2005). The advantage of DHs 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. DHs can, therefore, be multiplied for yield trials and agronomic evaluation much earlier than is possible with conventional practices for a self-pollinated crop plant (Houben et al., 2011). Accordingly, the use of DH lines has the potential to accelerate breeding cycles for production of homozygous lines and the release of new varieties. In several cereal crops, the production of DH lines is already an integral part in breeding programs to produce new varieties (Frisch and Melchinger, 2007). Haploid plants can also be *in vitro* generated from pollen (Tuvesson et al., 2003) or egg cells (Yang and Zhou, 1982) or from other cells of the gametophyte. After induced or spontaneous chromosome doubling, a doubled haploid cell is produced, which can be grown into a doubled haploid plant.

Table 1. List of examples on uniparental elimination of chromosomes after inter- or intraspecies hybridisation.

Female	male	retained genome	Reference
Avena sativa	*Zea mays*	*A. sativa*	(Matzk, 1996; Riera-Lizarazu et al., 1996; Rines and Dahleen, 1990; Rines and Dahleen, 1990)
A. sativa	*Pennisetum americanum*	*A. sativa* [1]	(Matzk, 1996)
Brassica napus	synthetic *B. napus*	*B. napus*	(Li et al., 2004)
B. rapa	*Isatis indigotica*	*B. rapa*	(Cheng et al., 2002; Tu et al., 2009)
Hordeum tetraploidum	*H. vulgare*	*H. tetraploidum*	(Bothmer et al., 1991)
H. bulbosum (2x)	*H. vulgare* (2x)	*H. vulgare* (x)	(Lange, 1971)
H. bulbosum (4x)	*H. vulgare* (4x)	*H. vulgare* (2x)	(Lange, 1971; Kasha and Kao, 1970)
H. jubatum	*H. bulbosum*	*H. jubatum*	(Rajhathy and Symko, 1974; Subrahmanyam and Bothmer, 1987)
H. jubaturn	*H. vulgare*	*H. jubaturn*	(Bothmer et al., 1991)
H. marinum ssp. gussoneanum	*H. vulgare*	*H. marinum ssp. Gussoneanum*	(Jorgensen and Bothmer, 1988)
H. marinum ssp. marinum	*H. vulgare*	*H. vulgare*	(Finch, 1983; Jorgensen and Bothmer, 1988)
H. parodii	*H. bulbosum*	*H. parodii*	(Subrahmanyam, 1977)
H. procerum	*H. bulbosum*	*H. procerum*	(Subrahmanyam, 1977)

H. procerum	*H. vulgare*	*H. procerum*	(Subrahmanyam, 1977)
H. vulgare	*Secale cereale*	*H. vulgare*	(Forster and Dale, 1983)
H. vulgare,	*T. aestivum*	*T. aestivum*	(Fedak, 1980; Molnar-Lang and Sutka, 1994)
H. lechleri	*H. vulgare*	*H. lechleri*	(Linde-Laursen and Bothmer, 1999; Linde-Laursen and Bothmer, 1993; Rajhathy and Symko, 1974)
H. vulgare (2x)	*H. bulbosum* (2x)	*H. vulgare* (x)	(Lange, 1971; Subrahmanyam and Kasha, 1973)
H. vulgare (4x)	*H. bulbosum* (2x)	*H. vulgare* (2x)	(Subrahmanyam and Kasha, 1973)
H. vulgare (2x)	*H. bulbosum* (4x)	*H. vulgare* (2x)	(Lange, 1971)
H. vulgare (4x)	*H. bulbosum* (4x)	*H. vulgare* (2x)	(Kasha and Kao, 1970; Lange, 1971; Subrahmanyam and Kasha, 1973)
H. vulgare	*Zea mays*	*H. vulgare*	(Chen et al., 1991)
Nicotiana tabacum	*N. africana*	*N. tabacum*	(Trojak-Goluch and Berbec, 2003)
N. tabacum	*N. plumbaginifolia*	*N. tabacum*	(Moav, 1961)
Solanum tuberosum	*S. phureja*	*S. tuberosum*	(Clulow et al., 1991)
Triticum turgidum	*Zea mays*	*T. turgidum*	(Almouslem et al., 1998; Dogramaci-Altuntepe and Jauhar, 2001)
T. aestivum	*H. vulgare*	*T. aestivum*	(Fedak, 1980; Molnar-Lang and Sutka, 1994)
T. aestivum	*H. bulbosum*	*T. aestivum*	(Inagaki and Snape, 1982; Sitch and Snape, 1986)
T. aestivum	*Z. mays*	*T. aestivum*	(Laurie and Bennett, 1986; Matzk and Mahn, 1994; Sarrafi et al., 1994)
T. aestivum	*Coix lachrymajobi*	*T. aestivum*	(Mochida and Tsujimoto, 2001)
T. aestivum	Teosinte (*Zea mays* spp. *Mexicana*)	*T. aestivum*	(Suenaga et al., 1998; Ushiyama et al., 1991)
T. aestivum	*Tripsacum dactyloides*	*T. aestivum*	(RieraLizarazu and Mujeeb-Kazi, 1993)
T. aestivum	*Pennisetum americanum*	*T. aestivum*	(Gernand et al., 2005; Matzk and Mahn, 1994)
T. aestivum	*Imperata cylindrica*	*T. aestivum*	(Komeda et al., 2007)
T. aestivum 'Chinese Spring'	*Sorghum bicolor*	*T. aestivum*	(Laurie and Bennett, 1988)
T. ventricosum	*H. bulbosum*	*T. ventricosum*	(Fedak, 1983)
Z. mays	*Z. mays*, haploid inducer lines	*Z. mays* [2]	(Coe, 1959; Eder and Chalyk, 2002; Kermicle, 1969; Zhang et al., 2008)

Notes:
[1] efficiency was too low for breeding programme,
[2] depends on the inducer line, paternal or maternal genotype could remain

1.3.1. Doubled haploid production in barley

For many years, doubled haploid spring and winter barley has been routinely used in breeding programs to achieve homozygosity from early generation (Kasha and Kao, 1970; Lange, 1971). The technique involves pollinating *Hordeum vulgare* with pollen of *Hordeum bulbosum* and, following fertilization; the *H. bulbosum* chromosomes are selectively eliminated leaving a haploid *H. vulgare* embryo which must be rescued and cultured on an artificial nutrient medium. Haploid plants which developed are colchicine-treated to double their chromosome numbers and to restore fertility (Jorgensen and Vonbothmer, 1988; Kasha and Kao, 1970; Thomas and Pickering, 1983).

A different approach to produce doubled haploid barley plants is anther and microspore culture (Devaux, 1987; Segui-Simarro and Nuez, 2008). The ‘‘bulbosum method’’ of obtaining doubled haploids for breeding programs has largely been superseded by anther culture, and latterly by microspore culture, as these methods can now be used routinely for regenerating doubled haploids more efficiently than the bulbosum method. However, the latter still has a role in developing mapping populations, as there appears to be less skewed segregation among the doubled haploid progenies than occurs in microspore culture-derived doubled haploids (Houben et al., 2011).

H. bulbosum is a perennial outcrossing species found in the Mediterranean region, and is in the secondary gene pool of *H. vulgare,* unlike *H. vulgare* it is a perennial plant (Bothmer et al., 1983). It normally requires vernalization to flower and has a strong self-incompatibility system based on two loci (Lundqvist, 1962). It occurs as two cytotypes, autotetraploid (2n = 4x = 28) and diploid (2n = 2x = 14), and has been crossed frequently with barley in attempts to transfer desirable characters from the wild into cultivated barley species (Gustavus et al., 1982; Johnston et al., 2009; Pickering and Johnston, 2005).

Kuckuck (1934) performed the first successful crosses between *H. vulgare* and *H. bulbosum* and obtained one sterile triploid hybrid plant. Later, Davies (1958) obtained three barley-like diploid plants from a cross between tetraploid *H. bulbosum* (female) and tetraploid *H. vulgare* (male) and suggested they originated by male parthenogenesis. Later reports of Kasha and Kao (1970), Symko (1969), and Lange (1969, 1971) independently presented the hypothesis of chromosome elimination as a mechanism of haploid barley production. This was confirmed by Kasha and Kao (1970) after hybridizations of diploid *H. vulgare* with diploid *H. bulbosum* resulted in production of haploid *H. vulgare* plants through complete loss of the *H. bulbosum* genome.

Once successful fertilisation of the *H. vulgare* egg has taken place, the *H. bulbosum* chromosomes are often eliminated during the first few mitotic divisions of the zygote, resulting in a haploid embryo, regardless of which parent is male or female. This is, however, greatly dependent on the ploidy level of the parental species and is strongly influenced by the parental genotypes and temperature during the early stages of embryo formation (Adamowski et al., 1998; Pickering, 1984).

Elimination could be alternative as uniparental elimination involving different parental genomes in different tissue of the same cross as in *H. marinum* × *H. vulgare*, elimination involved the *H. vulgare* genome in the endosperm, but the *H. marinum* genome in the embryo. In *H. vulgare* × *H. bulbosum* cross, the *H. bulbosum* genome was eliminated from both embryos and endosperms (Finch, 1983).

1.4. Effect of environmental and genotype factors on chromosome elimination

Haploid formation via chromosome elimination is known to depend on genetic factors (Ho and Kasha, 1975) and temperature after fertilization (Pickering and Morgan, 1985; Thomas and Pickering, 1983). In the cross of *H. vulgare* x *H.*

bulbosum in different conditions haploid *H. vulgare* or hybrid plant could be obtained. Temperature above 18ºC during the early stages of embryo growth can promote chromosome elimination while temperature below 18ºC favors stable hybrid.

In addition, the genotype of *H. bulbosum* is important as well. It has been reported that *H. bulbosum* genotypes from different sources can increase seed setting on incompatible cultivars of *H. vulgare,* embryo differentiation rates, and frequency of hybrid plants (Pickering, 1983), Cb 2920/4 and Cb 3811/3 are genotypes of *H. bulbosum* that have been used extensively for double haploid and stable hybrid production respectively (Pickering, 1983).

The genome balance between the parental species influences the chromosome stability too. Highest elimination happened in the genome ratio V:B (V for *H. vulgare* and B for *H. bulbosum*) is 1 or >1. If the genome ratio favors the *H. bulbosum* parent, then both parental chromosome complements are retained (Ho and Kasha, 1975; Kasha et al., 1971). When chromosome elimination occurs in the developing embryo it is rapid with 0-3 (and sometimes as many as 7) chromosomes per cell eliminated at each mitotic division (Bennett et al., 1976). Complete elimination of *H. bulbosum* chromosomes in embryos occurs 5–9 days after pollination (Bennett et al., 1976; Gernand et al., 2006; Subrahmanyam and Kasha, 1973). However, when elimination does not take place at this stage and hybrid plants are produced, elimination of *the H. bulbosum* chromosomes is slow and erratic in the somatic tissue.

Elimination may occur in some parts of the plant and not in others, it is called as “somatic chromosome elimination”. Subrahmanyam and Kasha (1973), Ho and Kasha (1975) found that parts of plants reverted to *H. vulgare* while other parts retained their hybrid morphology. Humphreys (1978) found different rates of chromosome elimination in different tillers of the same plant.

1.5. Mechanisms of uniparental chromosome elimination

Studies on the chromosomally unstable embryos show that chromosomes being eliminated have aberrant movement during mitosis and didn't assemble on the equatorial plate at metaphase, sister chromosomes failed to move toward the poles at anaphase and are retained in the cytoplasm to be eliminated from the nuclei (Gernand et al., 2005; Komeda et al., 2007). Lagging chromosome fragments enclosed during re-formation of nuclear membranes at the end of mitosis and formed micronuclei (Bennett et al., 1976; Fujiwara et al., 1997; Heddle and Carrano, 1977; Schubert and Oud, 1997).

Asynchrony at anaphase and bridge forming (Fujiwara et al., 1997), reduction in chromosome size and lacking of telomeric sequence (Fujiwara et al., 1997; Gernand et al., 2005), defect in attachment to spindle microtubules (Mochida et al., 2004), dicentric chromosome forming of unusual size (Fujiwara et al., 1997; Gernand et al., 2005; Kasha and Kao, 1970) in the genome undergoes elimination were observed as well. In addition eliminated chromosomes were less condensed and showed a snake-like structure (Gernand et al., 2006; Gernand et al., 2005).

In interphase, eliminating chromatin usually were spatially separated and tended to occupy distinct domains within the nuclei. Finally it was removed from the nucleus and formed micronuclei. This may reflect different interphase arrangements between these species combinations. Micronucleated chromatin undergoes heterochromatinization and DNA fragmentation (Gernand et al., 2005; Kasha and Kao, 1970). Figure 1 summarizes the processes of uniparental chromosome elimination in wide hybrids.

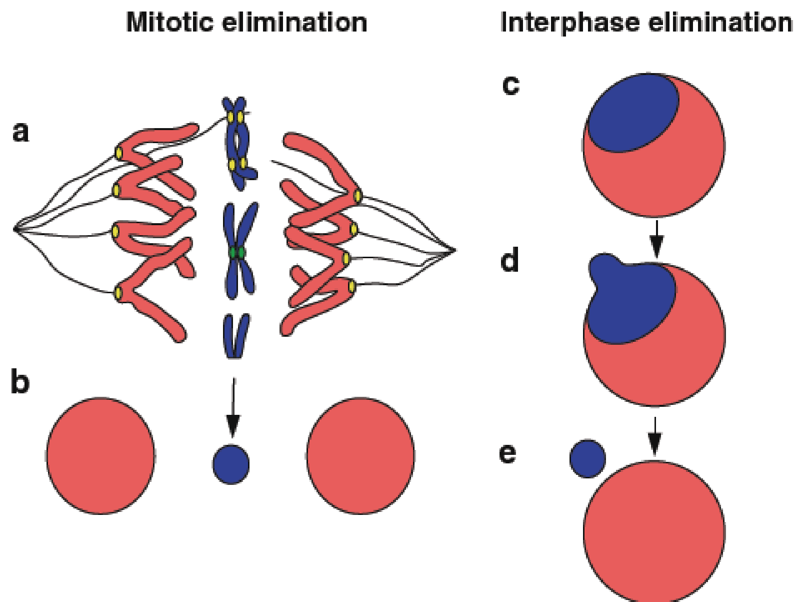


Fig. 1. Model for mitotic versus interphase elimination of chromosomes from unstable hybrid embryos. Mitotic elimination: (a) imperfect segregation of chromosomes caused by (1) faulty kinetochore/spindle fiber interaction, and (2) presence of additional or (3) absence of centromere; (b) formation of micronucleus and subsequent disintegration of micronucleus. Interphase elimination: (c) spatial separation of parental genomes, (d) ‘‘budding’’ of chromatin, (e) formation of micronucleus and subsequent disintegration of micronucleus (Houben et al., 2011)

Many hypothesis have been raised in an attempt to explain uniparental chromosome elimination, however, the exact mechanism is still obscure. For instance difference in timing of essential mitotic processes due to asynchronous cell cycling (Gupta, 1969) or asynchrony in nucleoprotein synthesis leading to loss of the most retarded chromosomes (Bennett et al., 1976; Laurie and Bennett, 1986), different time of chromosome replication (Gernand et al., 2005; Michel, 2000) and condensation (Bennett et al., 1976), imbalance between genetic factors of the two parents (Subrahmanyam and Vonbothmer, 1987), formation of multipolar spindles (Subrahmanyam and Kasha, 1973), difference in efficiency of parental chromosomes for attachment to the spindle proteins (Bennett et al., 1976), parent-specific inactivation of centromeres (Finch, 1983; Jin et al., 2004; Kim et al., 2002; Mochida and Tsujimoto, 2001) degradation of alien chromosomes by host-specific nuclease activity (Davies, 1974), kinetochore

inactivation (Laurie and Bennett, 1988), genetic disharmony between parental genomes and differences in cell cycle times (Bennett et al., 1976; Subrahmanyam and Kasha, 1973), incompatibility between the stable maternal cytoplasm factor(s) with the one(s) responsible for centromere separation of the paternal chromosomes (Fujiwara et al., 1997) and hybridization-mediated genomic shock (McClintock, 1984).

In the case of *H. vulgare* x *H. bulbosum* cross, it was suggested that during mitosis certain regions of the *H. bulbosum* chromosomes fail to replicate while *H. vulgare* chromosomes have a precocious replication. Such asynchrony could lead to bridges and breakage in the unreplicated regions of *H. bulbosum* chromosomes during division, resulting in the failure of these chromosomes to be included in daughter nuclei following cell division (Subrahmanyam and Kasha, 1973).

Further, it is suggested that chromosome elimination in *Hordeum* hybrids may be caused by a disturbed control of protein metabolism in hybrid seeds and perhaps *H. bulbosum* chromosomes are selectively eliminated because they are less efficient than *H. vulgare* chromosomes at forming normal attachments to spindle protein. After introduction of two different species that may have asynchrony in DNA synthesis, mitosis could lead to the preferential elimination of the chromosomes in which DNA synthesis is incomplete so chromosome of one species could fail to undergo normal congestion and anaphase migration and thereby become eliminated (Bennett et al., 1976). Chromosomes 2H and 3H of the *H. vulgare* parent seems to influence the chromosome stability in hybrids of *H. vulgare* and *H. bulbosum* (Ho and Kasha, 1975).

As the actual cellular mechanism involved in the process of uniparental chromosome elimination remains poorly understood the objective of this study was to investigate the processes of mitotic dependent selective elimination of

paternal chromosomes during the development of *H. vulgare* x *H. bulbosum* embryos.

In all cases of mitotisis dependent chromosome elimination, defects in chromosome segregation were reported (Fujiwara et al., 1997; Gernand et al., 2005; Kasha and Kao, 1970). As the centromere is essential for the correct chromosome segregation we thus hypothesised that uniparental chromosome elimination could be the consequence of a parent-specific defect in centromere functions.

1.6. Centromere structure

Accurate cell division requires the proper partitioning of chromosomes, resulting in daughter cells with the correct complement of genetic material (Ranjitkar et al., 2010). The centromere is essential to proper cell division, and it must be present on every eukaryotic chromosome. Centromeres are the chromosomal loci that direct the formation of the kinetochores. These macromolecular assemblies mediate the interaction between chromosomes and spindle microtubules and thereby power chromosome movement during cell division (Bernad et al., 2009).

Centromeres assemble from centromeric DNA/RNA that is packaged with histones and other kinetochore-related proteins to form a specialized type of chromatin (Choo, 2001). Although centromere proteins are well conserved among all organisms (Henikoff et al., 2000; Oegema et al., 2001), the DNA sequence organization at the centromere is not at all well conserved (Malik and Henikoff, 2002; Willard, 1998).

Individual organisms have evolved different genomic structures to create a locus capable of chromosome segregation. Centromeric DNA range in size and complexity from the 125 base pair point centromere found in budding yeast to the human centromere that spans several mega bases (Rudd et al., 2003) or even holocentric centromeres of *Caenorhabditis elegans* which span the entire length of chromosomes (Oegema et al., 2001).

Centromeric DNA are not only diverged in size but the sequences that make up the centromeres of diverse organisms are extremely variable (Choo, 2001), even centromeric DNAs within the same organism may vary among chromosomes (Lee et al., 1997; Willard et al., 1986) for example, organization of alpha satellite of human centromeres, varies from centromere to centromere (Lee et al., 1997). Lack of evolutionary conserved centromere-specific sequences and the conversion of noncentromeric regions into functional de novo centromeres have led to extensive speculation that centromeres are activated and maintained primarily by epigenetic mechanisms (Henikoff and Dalal, 2005; Houben et al., 2007; Karpen and Allshire, 1997).

1.6.1. Centromeric DNA

Centromeres of multicellular eukaryotes are often located in or near regions of repetitive DNA with an AT-richness greater than that of the genome average (Choo, 2001). Human centromeres are defined by the AT-rich repeats called alpha-satellite DNA (Willard, 1985). *Arabidopsis thaliana* has a 180-bp repeat family in the centromeric region of all five chromosomes and is arranged in a tandem manner that forms megabase-sized clusters (Hosouchi et al., 2002).

Gramineae species including rice have another type of repeat family in their centromeres referred to as centromeric retrotransposon (CR, that called "*cereba*" in barley) centromeric retrotransposons are often interspersed with the GC-rich satellites (Houben et al., 2007; Hudakova et al., 2001; Nagaki et al., 2005b; Wang et al., 2009; Zhong et al., 2002).

Repetitive DNA motifs are sharply diverged between species, making these repeats sequence unique for each species. Surprisingly, however, the presence of these repeats does not specify centromere location, and they are not required for the general function of centromeres (Black and Cleveland, 2011).

1.6.2. CENH3 and centromeric chromatin

Despite differences in nucleotide sequence, all centromeres share a unique chromatin composition that is characterized by the incorporation of the CENtromeric histone H3 (CENH3) within nucleosomes of centromeric chromatin. CENH3 is found at all active centromeres in a manner that appears to be independent of DNA sequence (Black et al., 2007) and has been proposed as the epigenetic mark of the centromere (Bernad et al., 2009).

CENH3 was initially identified in human (Earnshaw et al., 1985), it is known as CENP-A (CENtromeric Protein A) in mammals (Earnshaw et al., 1985; Li and Huang, 2008), CID in *Drosophila* (Henikoff et al., 2000), Cse4 in *Saccharomyces cerevisiae* (Stoler et al., 1995), Cnp1 in *Schizosaccharomyces pombe* (Takahashi et al., 2000), HCP-3 in *C. elegans* (Buchwitz et al., 1999) and HTR12 in *A. thaliana* (Talbert et al., 2002). In all likely models of centromere inheritance, CENH3 or its homolog is what physically distinguishes centromeric chromatin from the rest of the chromosome and is essential for the formation of a functional kinetochore in all eukaryotes (Black and Cleveland, 2011).

Several studies suggest that CENH3 stands at the base of the kinetochore assembly pathway, as almost all other kinetochore components are mislocalized in the absence of CENH3 (Blower and Karpen, 2001; Howman et al., 2000; Moore and Roth, 2001; Oegema et al., 2001). In most organisms, blocks of CENH3 nucleosomes are interspersed with histone H3 nucleosomes at centromeres (Blower et al., 2002). However, a single CENH3 nucleosome exists at the budding yeast centromere, consistent with a single microtubule-binding site (Furuyama and Biggins, 2007). Despite variation among organisms in the number of CENH3 nucleosomes at centromeres, the functions of CENH3 are conserved.

1.6.2.1. CENH3 properties

CENH3 has a conserved histone-folding domain at the C-terminal region and a N-terminal region that is quite variable between species (Henikoff et al., 2001). Since a N-terminal deleted CENH3 can be targeted to the centromeres, the N-terminal region is unnecessary for centromere targeting in *A. thaliana* (Lermontova et al., 2006) but it is necessary for the proper function of the centromere (Ravi et al., 2010).

The histone core domain comprises four helix domains (αN, α1, α2, and α3 helixes) and two loop domains (loop 1 and 2) (Black et al., 2004). A region in CENH3 defined as centromere targeting domain (CATD) is critical for centromeric localization of CENH3 in various species (Black et al., 2004; Vermaak et al., 2002). The CATD is composed of loop1 linker and α2 helix of CENH3 (Black et al., 2004; Sekulic et al., 2010) and its substitution enabled H3 chimera to incorporate into centromeres (Vermaak et al., 2002).This domain mediates molecular recognition events before and after nucleosome assembly and is important for binding of CENH3 to centromeric DNA (Black et al., 2004; Lermontova et al., 2006) to CENH3-specific chaperones (Dunleavy et al., 2009; Foltz et al., 2009; Shuaib et al., 2010), and to CENH3-stabilizing factors (Carroll et al., 2009; Lagana et al., 2010).

In contrast to invariant histone H3 (Rooney et al., 2002), CENH3 were shown to be evolved rapidly and positively selected among *Drosophila* (Malik and Henikoff, 2001), *Arabidopsis* (Talbert et al., 2002) and *Brassica* species (Cooper and Henikoff, 2004). Rapid evolution of CENH3 has been suggested to compensate for changes in centromere DNA that might result in unequal binding to spindle microtubules of centromere DNA variants (Henikoff et al., 2001; Malik and Henikoff, 2001).

1.6.2.2. CENH3 incorporation onto centromeric nucleosomes

As any other histone variant, the amount of CENH3 present at centromeres is diluted two fold during DNA replication, and must be replenished to maintain centromere identity (Bernad et al., 2009). Canonical histone H3 is synthesized during S phase and deposited by a chaperone complex containing Chromatin Assembly Factor-1 (CAF-1), which has been shown to bind to the DNA replication clamp to mediate physical coupling of histone H3 deposition to DNA replication *in vivo*. The constitutive histone variant H3.3 preferentially replaces and replenishes histones displaced by transcription and thus marks actively transcribed regions of the genome. H3.3 interacts with Histone cell cycle Regulation defective homolog A (HIRA), which mediates replication-independent chromatin assembly (De Koning et al., 2007; English et al., 2006).

CENH3 deposition onto centromeres is replication independent, it occurs during telophase/early G1 in human cells (Jansen et al., 2007), in early anaphase in *Drosophila* (Schuh et al., 2007), in a biphasic pattern during S and the G2 phases in *S. pombe* (Takahashi et al., 2005) and in late G2 phase in plants (*A. thaliana* and barley) (Lermontova et al., 2007; Lermontova et al., 2006).

Loading of *Dictyostelium* (species of soil-living amoeba) CENH3 occurs at the G2/prophase transition. This suggests that loading during G2/ prophase is the ancestral eukaryotic mechanism and that anaphase/telophase loading of CENH3 has evolved more recently after the Amoebozoa diverged from the animal linage (Dubin et al., 2010).

Recent studies have found many interacting partners of CENH3 which act as assembling/loading factors of CENH3 at the centromere in human, yeast or fruit fly (Aravind et al., 2007; Foltz et al., 2009; Furuyama et al., 2006; Shuaib et al., 2010), but the proteins involved in the loading of CENH3 in plants are unknown.

For instance in human, centromeric localization of Mis18 proteins (hMis18α and hMis18β) is required to recruit newly synthesized CENH3 to the centromere through regulating the acetylation status in the centromere, this "priming event" may involve protein acetylation, and protein-protein or protein–DNA interaction, which ensures the licensing of the centromere for later recruitment of CENH3 (Fujita et al., 2007).

In *C. elegans* (Maddox et al., 2007) and human (Fujita et al., 2007), disruption of a gene called Kinetochore NulL 2 (KNL-2 in *C. elegans* and Mis18BP1 in human) resulted in the dramatic loss of CENH3 from chromosomes resulting in a phenotype similar to CENH3 depletion itself (Oegema et al., 2001). Miss18 homologues are found in a complex with human KNL-2 (Fujita et al., 2007). It is likely that KLN-2 functions at centromere during loading, possibly as a targeting element for a CENH3 containing histone chaperone complex.

In human (Foltz et al., 2009), yeast (Sanchez-Pulido et al., 2009; Williams et al., 2009) and *Xenopus* (Bernad et al., 2011) Holliday Junction Recognition Protein (HJURP) was proposed as a cell cycle regulated CENH3-specific histone chaperone require for CENH3 chromatin assembly and stabilization of the prenucleosomal CENH3. In *Xenopus* additionally condensin II is required for the CENH3 assembling and retention in mitosis and interphase as well, it was suggested that condensin II enables CENH3 incorporation initiated by HJURP at centromere (Bernad et al., 2011).

In budding and fission yeast components of the canonical CAF-1 complex have been shown to be involved in CENH3 loading (Hayashi et al., 2004; Sharp et al., 2002) but it seems that it doesn't have a major role (Bernad et al., 2011). In fission yeast the chromatin remodeler Hrp1 (Walfridsson et al., 2005) and the histone binding protein NASP1-related protein Sim3 (Dunleavy et al., 2007) have been demonstrated to alter CENH3 assembly at the centromere. Sim3 interacts with non-chromosomal CENH3, possibly fulfilling a partially overlapping

chaperone role with HJURP. CAF1p48/RpAb48 (Mis16 in *S. pombe*) is a candidate for CENH3 chaperon in *Drosophila*, as it can assemble *Drosophila* CENH3 nucleosome along with H4 *in vitro* (Furuyama et al., 2006).

Remodeling and Spacing Factor (RSF) complex has been suggested to play a role in CENH3 assembly at the centromere of human. RSF can reconstitute CENH3 nucleosomes *in vitro* and its depletion induced loss of CENH3 and considered as a factor which consolidates CENH3 into a stable nucleosomal configuration (Perpelescu et al., 2009).

To maintain the identity of centromere, mechanism is needed that prevent CENH3 from stably incorporating into chromosome arms. In the budding yeast, ubiquitination by the E3 ligase Psh1, which specifically recognizes CENH3 through the CATD (Ranjitkar et al., 2010), triggers subsequent degradation of CENH3 at noncentromeric locations (Hewawasam et al., 2010; Ranjitkar et al., 2010).

Precisely after loading of CENH3 in human, a small GTPase switch functions to maintain newly assembled CENH3 nucleosomes to promote removal of spurious CENH3 (either excess at centromeres or outside true centromere loci). It modifies newly incorporated CENH3 and makes it identical to pre-existing CENH3 nucleosomes (Lagana et al., 2010). Figure 2 summarizes the loading pathway of the CENH3 in human cells as a model (Prendergast and Sullivan, 2010).

In human existing CENH3 nucleosomes was suggested to direct the incorporation of new CENH3 nucleosomes either directly or through the recruitment of intermediate factors that could include the covalent modification of surrounding centromeric chromatin. In turn HJURP must recognize either the existing CENH3 nucleosome or the intermediate factors or modifications that they induce in order to direct the deposition of new CENH3 nucleosomes only

into active centromeres (Foltz et al., 2009). Furthermore, in human depleted CENH3 could be compensated with Cse4 (budding yeast CENH3), and it was assumed that the residual of CENH3 may be sufficient to recruit ectopic Cse4 into centromeric nucleosomes (Wieland et al., 2004).

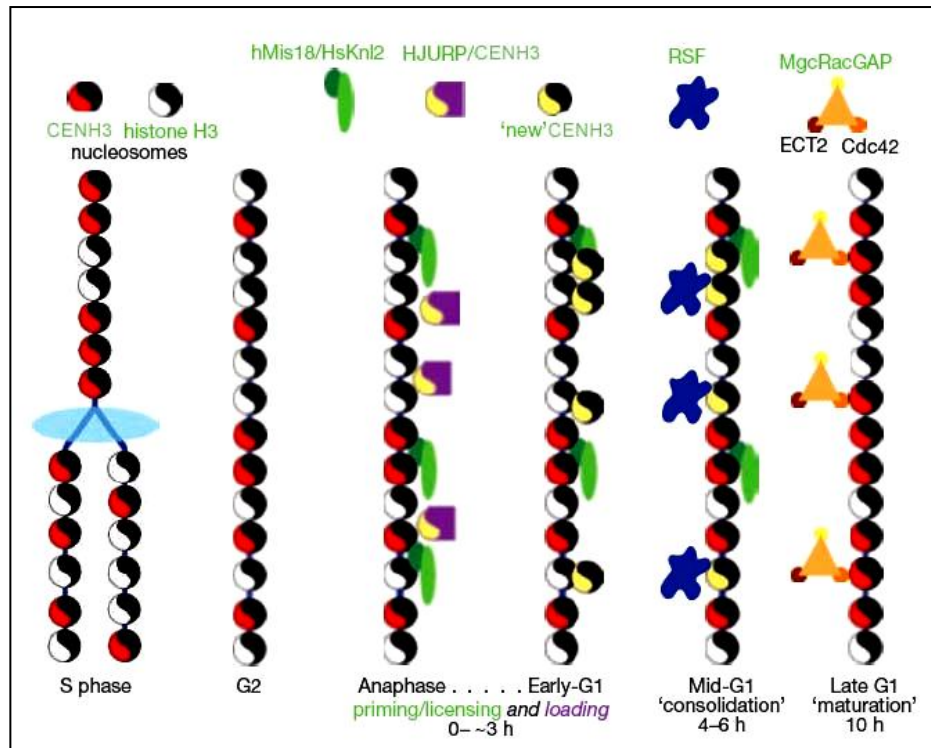


Fig. 2. Pathway for incorporation of new CENH3 in human. DNA replication in S phase (left) results in dilution of CENH3 at centromeric loci and presumed incorporation of normal histone H3- containing nucleosomes. This diluted configuration is the substrate for kinetochore formation in G2 phase of the cell cycle and mitosis. Immediately after anaphase, the hMis18–HsKNL2 complex (green) and HJURP (violet) associate with centromeres. The Mis18 complex 'primes' or licenses centromeric chromatin for CENH3 assembly, whereas HJURP acts as a CENH3-loading factor. In the following cycle (mid-G1), the RSF remodeling complex consolidates CENH3 into a stable nucleosomal configuration. Later, MgcRacGAP associates with centromeres to stabilize 'new' CENH3 at centromeric chromatin (Prendergast and Sullivan, 2010).

1.6.2.3. Other kinetochore proteins and their interaction with CENH3

The core centromere is comprised of proteins that are associated with CENH3 chromatin throughout the cell cycle referred to as the Constitutive Centromere-Associated Network (CCAN) that so far include, in addition to centromeric

nucleosomes, 15 proteins; CENP-C, CENP-H, CENP-I, CENP-K through CENP-U, and CENP-W (Cheeseman et al., 2008). Three different subsets of this collection of proteins are known as the CENH3 Nucleosome Associated Complex (CENH3 NAC) (Foltz et al., 2006), the CENP-H-I complex (Okada et al., 2006), and the interphase centromere complex (Izuta et al., 2006). Only NAC proteins were purified in associated with CENH3 nucleosomes, the rest could be purified in association with NAC (Foltz et al., 2006).

Among CCAN proteins, CENP-N is the first protein selectively bind CENH3, it was suggested that CENP-N interprets the information encoded within CENH3 nucleosomes and recruits to the centromeric chromatin other proteins required for centromeric function and propagation (Carroll et al., 2009). Several members of the CCAN have been shown to affect CENH3 levels at the centromere that include CENP-H, CENP-I, CENP-K, CENP-M (Okada et al., 2006) and CENP-N (Carroll et al., 2009). Mutations that affect CENP-N result in loss of centromere function and affect CENH3 level at centromere. This defect results from an inability to assemble or stabilize newly synthesized CENH3 (Carroll et al., 2009). CENP-H-I complex in human were showed to contribute to the efficient incorporation of newly synthesized CENH3 into centromere as newly expressed CENH3 is not efficiently incorporate into centromere in knockout mutant of a subclass of CENP-H-I (Okada et al., 2006).

In flies, another member of this complex, CENP-C is also required for maintaining CENH3 levels at the centromere, although this requirement appears specific for this species (Erhardt et al., 2008). It was reported that condensin defection in human (Samoshkin et al., 2009), budding yeast (Yong-Gonzalez et al., 2007), fruit fly (Jager et al., 2005) and in *Xenopus* (Bernad et al., 2011) disrupt the localization and maintenance of the CENH3 that could be cause of stretching in metaphase and increase in loss of rigidity in kinetochore.

1.7. Centromeres of *H. vulgare* and of *H. bulbosum*

In cereal species, two families of repeats are conserved in the centromeric regions. One is the Cereal Centromeric Sequence (CCS1) family of *Brachypodium* that also occurs in wheat, rye, barley, maize and rice centromeres (Aragon-Alcaide et al., 1996). The other family is the *Sau3A9* repeat sequence of sorghum which also hybridized to the primary constriction of the above species (Jiang et al., 1996).

Barley homologue of CCS1 represents a part of the Long Terminal Repeats (LTRs) of a Ty3-*gypsy* retrotransposon (Presting et al., 1998) and Sau3A9 sequence is partially homologous to the sequence of the integrase gene in the polyprotein-coding region of a Ty3-*gypsy* retrotransposon (Miller et al., 1998).

The centromeric DNA of *H. vulgare* and of *H. bulbosum* is composed of the, centromeric retrotransposon *cereba*, a Ty3/gypsy-like retroelement. *H. vulgare* centromeres are further marked by a GC-rich satellite sequences $(AGGGAG)_n$ (Hudakova et al., 2001). Single barley chromosomes contain an average of about 200 *cereba* elements (Presting et al., 1998; Hudakova et al., 2001). Further it was shown that the TC/AG-repeat microsatellite sequence derived from the rice blast fungus (*Magnaporthe grisea*) hybridized to all of the centromeres of *H. vulgare* chromosomes, but not to *H. bulbosum* centromeres (Kim et al., 2002). Chromatin fiber immunolabeling and FISH experiments clearly demonstrated only a portion of the centromeric repeats interacts with CENH3 of barley (Houben et al., 2007).

2. Aim of the thesis

In this study I aimed to investigate the role of CENH3 in the process of selective elimination of paternal chromosomes during the development of *H. vulgare* x *H. bulbosum* hybrid embryos. The main questions that I tried to answer in this project were:

1- Are both parental CENH3s active in unstable hybrid embryos or does parental-specific inactivation of CENH3 occur? To test this I cloned and sequenced both parental CENH3s and investigated their transcription in unstable and stable hybrid embryos.

2- What is the situation of CENH3 incorporation in stable species combinations? To test this we generated barley CENH3-specific antibodies and performed indirect immunostaining on three different genotypes: a stable hybrid of *H. vulgare* x *H. bulbosum* plants (to investigate CENH3 incorporation when both genome CENH3s present in combination), a *H. vulgare*/ *H. bulbosum* 7H substitution line (CENH3 incorporation when one of the parental CENH3 presents) and a *T. aestivum*/ *H. vulgare* 1H+6H addition line (CENH3 incorporation in combination of less related species).

3- Is the centromeric loading time of CENH3 similar in both studied parental species? I used indirect immunostaining with anti-CENH3-specific antibodies on fractionated nuclei of each parent and their stable hybrid.

4- We found that *H. vulgare* and *H. bulbosum* species encode each two CENH3 variants. To characterize the chromosomal distribution of both CENH3 variants,-specific antibodies were generated for each variant of *H. vulgare* CENH3. Double immunostaining experiments were performed on cells undergoing meiosis or mitosis and on extended chromatin fibers.

3. Material and methods

3.1. Plant growth conditions and crossing procedures

Two genotypes of *H. bulbosum* (2920/4 and 3811/3) were vegetatively propagated and vernalized for 7-8 weeks at 4°C, 8 h day length. Vegetative propagation is necessary since *H. bulbosum* is self-incompatible (Bothmer et al., 1995) and individual genotypes cannot be established from seed. After vernalization, the two genotypes were maintained separately in cool glasshouses (temperatures <18°C) with 16 h day length. After flowering the plants were cut back and vernalized again to complete the life-cycle.

For the *H. vulgare* (cv. 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 cultivated until ear emergence in a cool glasshouse and then transferred to their respective environments.

Crossing was done conventionally by emasculating florets of the female parent prior to anthesis, the spikes covered with bags to prevent out-pollination and pollinated with freshly collected pollen from the male parent. A post-pollination application of plant growth regulators was done one day (summer) or one and two days (winter) after pollination to stimulate seed development and improve the quality of the seeds. The mixture comprised 75mg/L gibberellic acid (GA3) + 1 mg/L dicamba, with or without 2mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Twelve drops/L of Tween 20 was added as a surfactant. Immature embryos of various sizes were excised under a stereomicroscope for further analysis.

3.2. Plant material used for CENH3 gene mapping

To determine which barley chromosome carries CENH3 genes, seven wheat-barley addition lines (1H+6H, 2H, 3H, 4H, 5H, 6H and 7H) were utilized using barley CENH3 type-specific primers (primer 6 and 16 for αCENH3 and 4 and 8 for βCENH3). The material was provided by S. Nasuda (Kyoto, Japan).

To determine which *H. bulbosum* chromosome carries CENH3 genes, *H. bulbosum* CENH3-specific primers (primer 17 and 18 for αCENH3 and primer 4 and 7 for βCENH3) were used on different barley-*H. bulbosum* substitution lines (2H, 3H, 4H, 5H, 6H and 7H), and a barley line (916J2/2) with big introgression of 6HL (half of the arm) and small distal introgression on the 6HS of *H. bulbosum* (Table 2). The material was provided by R. Pickering (The New Zealand Institute for Plant and Food Research Limited, Christchurch, New Zealand).

Table 2. List of *H. vulgare* lines with *H. bulbosum* introgressions/substitutions.

Nr.	Line code	Chromosome constitution
1	916J2/2	Introgressions on 6HL (0.5 of the arm) and introgression on 6HS (small distal)
2	919Q4	6H *H. bulbosum* chromosome substitution
3	16R5	6H *H. bulbosum* chromosome substitution and introgression on 7HL
4	38M1	6H *H. bulbosum* chromosome substitution
5	38M11	6H *H. bulbosum* chromosome substitution
6	36L46	6H *H. bulbosum* chromosome substitution
7	333Y1	Introgressions on 1HL (small distal, two spots-band)
8	315A1	Introgressions on 1HL (medium distal)
9	230H1	Introgressions on 1HS (distal large) and 5HL (small distal)
10	181P267/M2/M1/M2/1/1pl2	Introgressions on 6HL distal, 15-20% of arm

3.3. Extraction of plant RNA

3.3.1. RNA isolation from plants

Seeds of *H. vulgare* were germinated on moist filter paper at room temperature in the dark and ~5 cm-long roots and ~4 cm-long coleoptiles were collected for RNA isolation. RNA of *H. bulbosum* was prepared from young spikes. Total RNA was extracted with Trizol method (Chomczynski and Sacchi, 1987a, b). The integrity and quantity of RNA was assessed on a 1% denaturing MOPS-formaldehyde electrophoresisagarose gel (Sambrook et al., 2001).

3.3.2. RNA isolation from young embryos

Embryos of different ages (4-15 days after pollination (DAP)) were pooled (3-5 embryos per sample) and transferred into 100 ul RNAlater-RNA Stabilization Reagent (Qiagen) and stored in -20ºC till use. RNA was extracted using PicoPure RNA Isolation Kit (Arcturus).

3.4. cDNA synthesis and reverse transcription-PCR (RT-PCR)

The RNA concentration was determined spectrophotometrically. To remove residual DNA contamination RNA samples were treated with RNase-free DNase (Fermentas); extracted RNA from embryo was treated with RNase-Free DNase Set (Qiagen, that is suitable for on-column treatment). cDNA was prepared using the Reverse Aid H Minus First Strand cDNA Synthesis Kit (Fermantas) according the company's instructions.

3.5. RT_PCR

PCR was done on PCR mixed contained 6 pmol of each primers (Table 3), 0.2 mM dNTP, 1x reaction buffer (contains $MgCl_2$), 1x Q-solution and 1U Taq polymerase (Qiagen). PCR conditions included following steps: 94°C for 3', 40 cycles: 94°C for 1', annealing temperature for 1':10'' and 72°C for 1':30''. The annealing temperature used is given in table 3.

3.5. Race PCR

To obtain the missing sequences of CENH3 genes the SMART™ RACE cDNA Amplification Kit (Clontech Company) was used following company's instruction. Therefore, total RNA was extracted with Trizol method from roots. 100 µg of total RNA was used for polyA mRNA isolation using the Dynabeads® mRNA DIRECT™ Kit (Invitrogen). cDNA was prepared using the Reverse Aid H Minus First Strand cDNA Synthesis Kit (Fermantas) according the company's instructions.

3.6. Cloning, sequencing and analysis of PCR fragments

DNA fragments were ligated into the pGEM-T easy vector (Promega). The ligation product was electroporated in *E. coli,* DH5α (Stratagene). Transformants were selected on LB agar supplemented with ampicillin (100 µg/ml), 5-bromo-4-chloro-3-indolyl-β-D-GALactopyranoside (X-Gal) (64 µg/ml) and IsoPropyl-β-D-ThioGalactopyranoside (IPTG) (0.2 mM). White colonies were individually cultured in liquid LB overnight. Plasmid DNA was extracted using a 'miniprep' protocol, digested with *Eco*RI (20 units, 37°C, 12 hours) and colonies which had inserts of the expected size have been sequenced using the PGRC Sequencing Service (IPK, Gatersleben). Sequences were analyzed and aligned using the software Lasergene (DNASTAR).

3.7. Extraction of genomic plant DNA

Plant genomic DNA (gDNA) was extracted from 100 mg grinded leaves in 15 ml prewarmed at 65ºC 2x CTAB-buffer (0.1 M Tris, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB, 0.5% Na-bisulfade, 1% 2-mercaptoethanol) for 1 hour. After adding 20 ml isoamylalcohol:chloroform (1:24) the mix was centrifuged at 8,000 rpm, at room temperature. The supernatant was transferred into a new tube, the DNA precipitated with 14 ml isopropanol, the pellet washed with cold 70% ethanol, then dried and resuspended in 500 µl double distilled H2O (Doyle and Doyle, 1990).

3.8. Cleaved Amplified Polymorphic Sequence (CAPS)

PCR product were purified using PCR Purification Kit (QIAquick) according the company's instructions. Around 600 ng of purified PCR product were digested using *Alw*I (Fermentas), 55ºC for 5 h or *Ban*II (Fermentas), 37ºC for 8 h. Digested PCR products were size-fractionated by gel electrophoresis and recorded.

3.9. Indirect immunostaining

3.9.1 Slide preparation

Immunostaining was done on slide prepared from root tip, embryo, isolated leaf nuclei or stretched chromatin fiber

3.9.1.1. Slide preparation from root tip

Seeds were germinated on moist filter paper at room temperature in dark for 3 days. Root tips (1.5 - 2 cm) were treated with ice cold water for 17 hours to synchronize cell divisions, then fixed for 20 minutes at room temperature and 25 minutes on ice in freshly prepared 4% paraformaldehyde solution (PFA) containing phosphate-buffered saline (1xPBS, pH 7.3), washed three times for 15 minutes in 1x PBS on ice. Meristematic regions were digested by treating with an enzyme mix (2.5% pectinase, 2.5% cellulase 'Onozuka R-10', 2.5% pectolyase Y-23 dissolved in 1x PBS) at 37ºC until the material become soft (about 50 minutes). The macerated material was washed and then squashed in PBS. Coverslips were removed using liquid nitrogen and slides were immersed in 1x PBS. For longer storage the slides were kept in 100% glycerol at 4°C. For immunostaining with anti-tubulin antibody, material was fixed in 4% paraformaldehyde solution containing microtubules stabilizing buffer (1xMTSB prepared with 50 mM PIPES, 5 mM MgSO4, 5mM EGTA).

3.9.1.2. Slide preparation from embryo

Young embryos were isolated and fixed for 15 minutes in 3% PFA at room temperature, washes in 1xPBS and treated with 1% of enzyme mix for 10-15 minutes at 37ºC (depends of the size of embryo). After washing in PBS embryo were squashed on coated slides (polysine microscope slides, Thermo scientific) and coverslips were removed using liquid nitrogen.

3.9.1.3. Slide preparation from leaf isolated nuclei

Nuclei were isolated from young leaves or root tips. Therefore, plant material was fixed in 4% paraformaldehyde solution containing phosphate-buffered saline (1xPBS, pH 7.3) for 20 minutes in vacuum then washed two times for 10 minutes each time in tris buffer (10mM tris, 10mM Na_2EDTA, 100mM NaCl and 0.1% Triton X-100, pH 7.3). Fixed sample was chopped with razor blade in isolation buffer (15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermin, 80 mM KCl, 20 mM NaCl, 15 mM mercaptoethanol and 0.1% Triton X-100). Cell suspension was filtered and spin onto a slide using cytospin (400 rpm for 5 minutes). After, slides were immersed in 1x PBS. For longer storage the slides were kept in 100% glycerol at 4°C.

3.9.1.4. Preparation of stretched chromatin fibers

Nuclei were isolated from young etiolated leaf material and chopped with a razor blade in Galbraith buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS and 1%Triton X-100) (Galbraith et al. 1983). Nuclei were filtered and spin into a slide using cytospin (700 rpm for 5 minutes). After, nuclei were treated with 100 µl lysis buffer (25 mM Tris (pH 7.5), 500 mM NaCl, 1% triton X-100 and 0.2 urea (Jin et al., 2004) and covered with parafilm. After 15 minutes the parafilm was slowly dragged down the slide to stretch chromatin and the preparation was fixed in 4% paraformaldehyde in 1X PBS for 10 minutes and later immersed in 1x PBS.

3.9.2. Indirect immunofluorescence

The stored slides in glycerol were washed 3 times for 10 minutes with 1X PBS buffer. To avoid non-specific antibody binding, slides were blocked with MAXblock™ Blocking Medium (Active Motif) in a moist chamber at room temperature for 2 hours. After 5 minutes washing in 1X PBS, 50 µl of primary antibody (diluted 1:100 in 1x PBS, 2% BSA, 0.1% Tween 20) were applied to each slide and covered with parafilm and incubated in a moist chamber overnight at 4°C. Then slides were washed 2x10 minutes in 1x PBS and a secondary antibody was applied, covered with parafilm and incubated for 1 hour at 37°C in a humid chamber in dark. After washing the slides 3x 10 minutes in 1% PBS, chromosomes were counterstained using 50 µl of antifade containing 10 µg/ml DAPI (4´, 6-DiAmidino-2-Phenylindole). Samples were analyzed using a fluorescence microscope BX61 (Olympus). To observe each fluorescence-specific filters were used DAPI filter (excitation wavelength 359 nm, emission wavelength 461 nm), FITC filter (excitation wavelength 490 nm, emission wavelength 525 nm) or rhodamine filter (excitation wavelength 540 - 560 nm, emission wavelength 580 nm). The pictures were taken by cooled sensitive charged-couple device (CCD) camera. Deconvolution microscopy was employed for superior optical resolution of globular structures thus each photograph was collected as sequential image along the Z-axis with approximately 10 slices per specimen. All images were collected in gray scale and pseudocolored with Adobe Photoshop.

3.10. Generation of CENH3-specific antibodies

Polyclonal IgG antibodies were generated specific for CENH3s of barley. Therefore, suitable epitopes were identified and peptides synthesized. Peptide synthesis, immunization of animals and affinity purification of antiserum was performed by Pineda, Antikörper-Service.

3.11. Protein extraction

Proteins were extracted from leaves and isolated nuclei.

3.11.1. Total protein extraction from leaves

200 to 300 mg of leaf sample was ground under liquid nitrogen and suspended in 1 ml of solubilisation buffer (112 mM Na_2CO_3, 112 mM DTT, 4% SDS, 24% sucrose, and 4 mM EDTA). After 20 minutes of incubation at 65°C, cell debris was removed by centrifugation. Total protein was transferred to a new tube and stored at -20°C.

3.11.2. Protein extraction from isolated nuclei

Nuclei were isolated from etiolated young leaves. 20 g of leaf sample were ground in liquid nitrogen and suspended in 10 ml of 1X TBS (10 mM Tris (pH 7.5), 3mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM PMSF (Phenyl Methyl Sulfonyl Fluoride)) with protease inhibitors (Complete; Roche Molecular Biochemicals) and 0.5% Tween 20. Nuclei were filtered through one layer of Miracloth and then purified using 25/50% sucrose gradient, then centrifuged and nuclei pellet was used directly for protein isolation.

3.12. Western blot analysis

The protein samples were quantified by Bradford assays (Bradford, 1976), and 40 µg of protein of each sample were separated on 10% SDS-PAGE polyacrylamide gels on a Miniprotean II (Bio-Rad) using Tricin-SDS-PAGE running buffers (Laemmli, 1970). Proteins were blotted on a nitrocellulose membrane by a Trans-Blot semi-dry apparatus according to Bio-Rad manual instruction for 2 hours at 20V in blotting buffer (48 mM Tris, 39 mM glycine, 20% methanol, 1.3 mM SDS, pH 9.2). Protein loaded membrane was blocked for 1 hour in blocking milk (5% fat free dry milk in PBS + 0.1% Tween 20), and incubated overnight at 4°C with the primary antibody (1:1000) in blocking milk. The secondary antibody (anti-rabbit IgG: IRDye800 conjugated, LI-COR, diluted 1:5000 or anti-guinea pig IgG: horseradish peroxidase conjugated, Dianova,

diluted 1:5000) was applied for 2 hours at room temperature. After 3 times washing with PBST (PBS buffer + 0.1% Tween 20), 10 minutes each time, then for IRDye conjugated antibody, fluorescence signal was detected with the ODYSSEY imaging system (Li-COR) and for horseradish-proxidase conjugated antibody, signals were enhanced with mixture of enhancer and peroxide solutions (1:1) for 5 minutes. Signals were then visualized using hyperfilm ECL (GE Healthcare).

3.13. Fluorescence *in situ* hybridization (FISH)

3.13.1. Preparation of chromosome spreads using drop technique

Caryopses were germinated on moist filter paper at 22-24ºC in dark during 3-4 days. Root tips (1.5 - 2 cm) were treated with ice cold water for 17 hours for *H. vulgare* and 20 hours for *H. bulbosum* to accumulate metaphases, then fixed in ethanol: acetic acid (3:1) for 2-3 days at room temperature. Root tips were washed with ice water for 2 minutes twice and with citrate buffer (8.2 µM citric acid, 1 µM sodium citrate pH 6) for 5 minutes twice. To remove the cell walls the root meristems were treated with an enzyme mixture (2.5% pectinase, 2.5% cellulase 'Onozuka R-10', 2.5% pectolyase Y-23 dissolved in 1x PBS) at 37ºC until the material become soft (about 50 minutes) and washed with citric buffer and 96% ethanol. Tissue was broken with dissecting needle in freshly prepared methanol: acetic acid (1:3) and was dropped on cold slides according to (Kato et al., 2006). The preparation was checked under a phase contrast microscope.

3.13.2. Probe preparation for FISH

Probes were labeled with Atto-590-dUTP or Atto-488-dUTP (Jena bioscience) by nick translation following company's instruction. After, probes were precipitated by adding 1/10 volume 3 M sodium acetate and 2.5 volume ice-cold 95% ethanol and kept for overnight at –20°C. After centrifugation the precipitate was washed with 0.5 ml 70% ethanol, dried and resuspended in 20 µl water.

3.13.3. *In situ* hybridization

Before hybridisation the slides were cross-linked using UV irradiation (120 mJ/cm^2) for 15 seconds and fixed in 4% paraformaldehyde (in 2X SSC) at room temperature for 10 minutes, washed for 2x 5 minutes with 2x SSC and dehydrated for 2 minutes in an ethanol series (70, 90 and 100 %) and air dried. 50 ng of labeled probes were applied in 30-40 µl of hybridization mix (50% deonized formamide and 10% dextran sulfate in 2x SSC) per slide. For genomic *in situ* hybridization (GISH), gDNA of *H. bulbosom* was sonicated and labeled as described. 120 ng of probe with 60 times more of blocking DNA (sonicated *H. vulgare* gDNA) was dissolved in 20 µl of hybridization mix. The hybridization mixture and the chromosomes were denatured together on a heating plate at 80°C for 2 min and incubated in a moist chamber at 37°C overnight. Post-hybridization washing was carried in 2×SSC at 60°C for 20 minutes. After dehydration in an ethanol series (70, 90 and 96%), the slides were air dried and counterstained with 4',6-diamidino-2-phenylindole (DAPI) in antifade. Samples were analyzed using a fluorescence microscope BX61 (Olympus). To observe each fluorescence-specific filters were used DAPI filter (excitation wavelength 359 nm, emission wavelength 461 nm), FITC filter (excitation wavelength 490 nm, emission wavelength 525 nm) or rhodamine filter (excitation wavelength 540 - 560 nm, emission wavelength 580 nm). The pictures were taken by cooled sensitive charged-couple device (CCD) camera. All images were collected in gray scale and pseudocolored with Adobe Photoshop.

3.14. Cell-free translation

Templates were prepared by PCR with the primer pairs 21/22 for αCENH3 and 2/13 for βCENH3, adding the T7 promoter and terminator. The PURExpress cell-free transcription-translation system (New England Biolabs) was used for *in vitro* protein synthesis. Briefly, a 250 ng template was combined on ice with 12.5 µl of solution A and 5µl of solution B along with RNase inhibitor and then the mixture was incubated at 37 °C for 1 hour. Samples were then directly analyzed by Tricine-SDS-PAGE.

Table 3. List of primers.

Nr.	Name of primers	Sequence 5'-----3'	Annealing temperature in °C
1	αCENH3-F	ATGGCCCGCACCAAGCACCCCG	71.99
2	βCENH3-F	ATGGCTCGCACGAAGAAAACGG	64.54
3	αCENH3-R	CAGTGCCACCGTGCCTGGCCTG	71.99
4	Race-CENH3-F	GTGGCCACTGCGGGAGATCAGGAAGTACC	71.94
5	Degenerate-F	GTRGCRCTGCGGGAGATCAGGA	68
6	H.vαCENH3-gDNA-R	GTGCAAACGGGATGAGAAAATT	58.94
7	H.bβCENH3 -R	ATGGCGTCGGCTTGTTGGACCC	67.98
8	H.vβCENH3-R	GTCGGCTTGCTCTCCTTCTTGTTCG	67.86
9	H.b-αCENH3-R	TTGGGGAGTCCAGCGGCTGATTGC	69.69
10	H.v αCENH3-R	CAGCGTTTGACTCGAGGACAGTAG	66.28
11	Race-CENH3-R	TCCTTTTGCATGACGGTAACACGCT	69.01
12	Degenerate-R	CTBGCRAGYTGYATGTCCTTTT	61
13	βCENH3- 3'-R	GCAAAGGCCGAGAAGTCAGATG	64.54
14	T.a-α+βCENH3-R	GTTCATCTCGCCCTTCGTCSAGGTC	67.98
15	T.a-θCENH3-R	GCGGCTGATTTCAGGACAGAAG	64.54
16	H.vαCENH3-gDNA- F	AGAAGAAGATCGGGTCCGGCTA	64.54
17	H.v/H.b αCENH3-F	CGGGCACGTCCGAGACTCC	68.79
18	H.v/H.b αCENH3-R	GTAGAATTCGGTGACCTCCTTGACC	66.22
19	GAPDH-F	CAATGCTAGCTGCACCACCAACTG	58.8
20	GAPDH-R	CTAGCAGCCCTTCCACCTCTCCA	58.8
21	αCENH3-F-2	ATGGCCCGCACCAAGCACCC	65.5
22	αCENH3-entire-R	GTAACACGCTTTGCATGGATGGCACAG	66.5

4. Results

4.1. CENH3 of *H. vulgare* and *H. bulbosum* cross-reacts with an anti-grass CENH3-specific antibody

In order to detect the CENH3 protein in *H. vulgare* x *H. bulbosum* hybrid embryos an anti-grass CENH3 antibody (Nagaki et al., 2004) was tested first separately on *H. vulgare* and *H. bulbosu*m to ensure cross-reactivity. To determine the antibody specificity, indirect immunostaining was performed on mitotic root cells of *Hordeum* species and wheat. In addition, a protein gel blot assay on proteins isolated from mature leaves of *H. vulgare* and *H. bulbosum* was conducted.

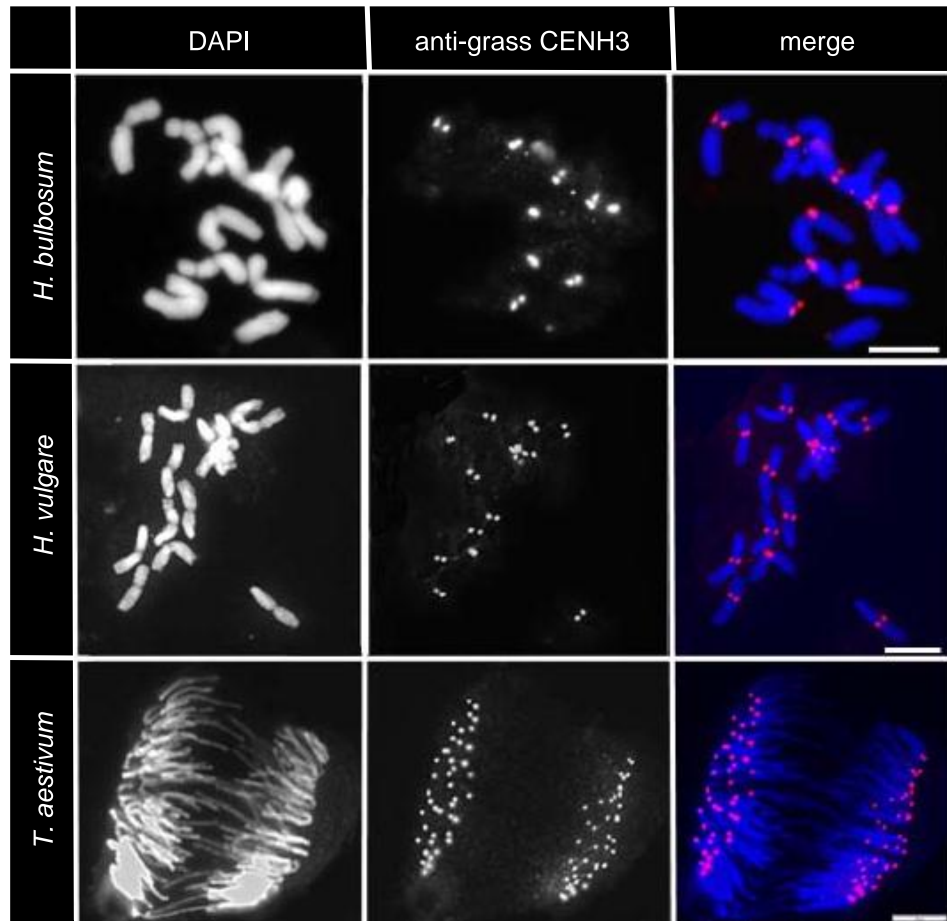


Fig. 3. Indirect immunostaining using an anti-grass CENH3 antibody identifies centromeres in *H. vulgare, H. bulbosum* and wheat (CENH3-specific signals are in red). Chromosomes were counterstained with DAPI (in blue). Scale bar present 10µm.

H. vulgare (2n=2x=14) and *H. bulbosum* (2n=2x=14) displayed each up to 14 centromere--specific signals after immunostaining. Wheat (2n=6x=42) showed 42 centromere-specific CENH3 signals (Fig. 3). The Western blot assay using the grass CENH3 antibody revealed two bands (Fig. 4). The major band corresponds with the position of Rubisco protein. The minor band, with a size less than 25 kDa, corresponds to the predicted size of the CENH3 protein. Hence, Western blot analysis and indirect immunostaining showed that the anti-grass CENH3 antibody cross-reacts with the corresponding proteins in both *Hordeum* species and wheat.

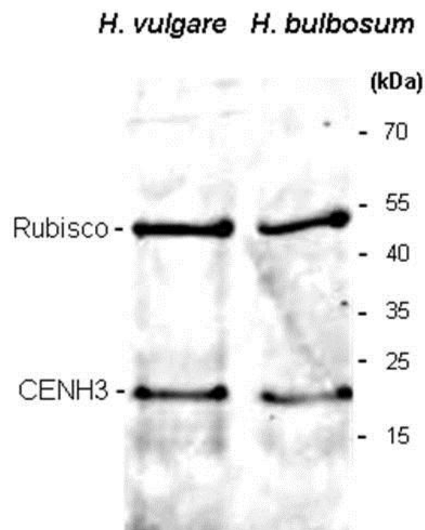


Fig. 4. Western blot experiment using an anti-grass CENH3 antibody. Total proteins were extracted from leaves of *H. vulgare* and *H. bulbosum*. Appropriate CENH3 protein band should be located at less than 25 kDa.

4.2. Lagging chromosomes in unstable *H. vulgare* x *H. bulbosum* hybrid embryos are CENH3-negative

Next, immunostaining experiments with grass CENH3 and alpha-tubulin-specific antibodies were conducted on unstable hybrid embryos to elucidate whether the centromeres of the *H. bulbosum* chromosomes are functionally. Indirect immunostaining of anaphase cells of few days old unstable hybrid embryos revealed that the centromeres of segregated chromatids were decorated with CENH3-specific signals. Lagging chromosomes did not reveal CENH3 signal

(arrowed in Figure 5A). In this cross, *H. bulbosum* chromosomes are lagging behind *H. vulgare* chromosomes and the sister chromatids of *H. bulbosum* segregate asymmetrically at anaphase (Fig. 6) (Gernand et al., 2006). However in stable hybrid, *H. bulbosum* chromosomes segregate normally and are CENH3-positive (Fig. 5B).

A clear interaction between tubulin fibers and kinetochores is not detectable due to the limited optical resolution. The primary constriction of lagging chromosomes is not recognizable. Absence of CENH3 suggests centromere inactivity resulting defect in microtubule attachment, missegregation and elimination of chromosomes via micronuclei formation.

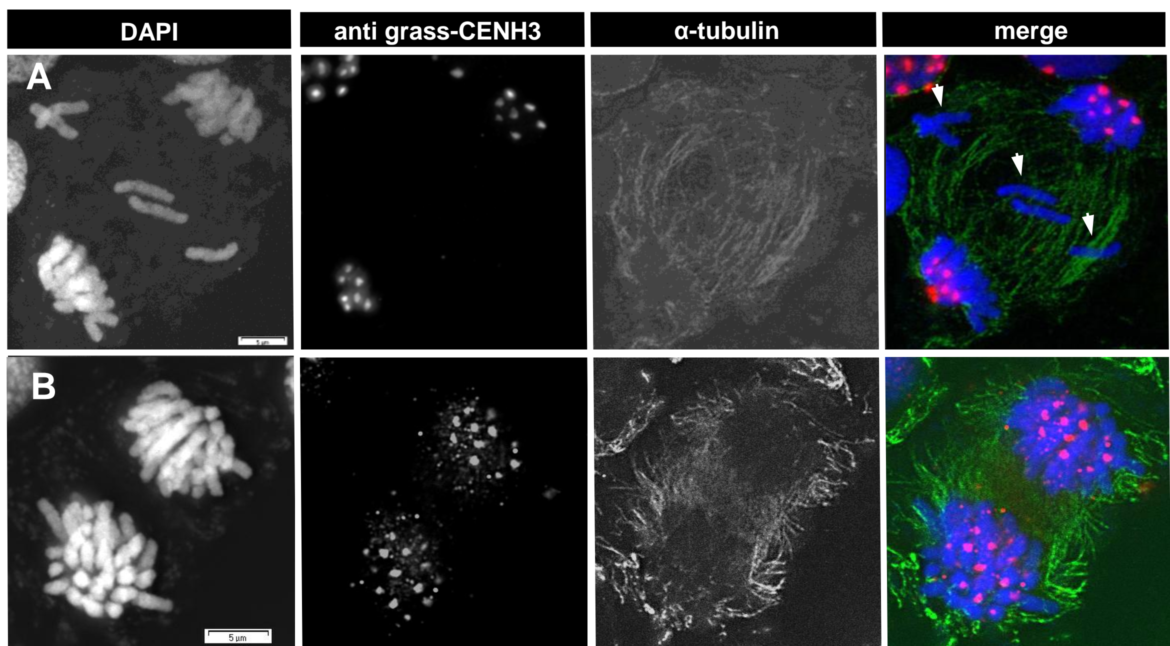


Fig. 5. Anaphase cell prepared from (A) a 5 day old unstable *H. vulgare* x *H. bulbosum* embryo, (B) stable hybrid embryo as control, after immunostaining using grass CENH3- (in red) and tubulin (in green)-specific antibodies. Chromosomes are counterstained with DAPI (in blue). Lagging chromosomes (arrowed) show no CENH3 signals suggesting an important role of CENH3 in chromosome elimination. Scale bar presents 5 μm.

In addition, in unstable hybrid embryos some nuclei with more than seven CENH3-signals displayed immunosignals of different intensity and size. The number of weak CENH3-signals was seven or less and strong immunosignal were seven or more. It is likely that the centromeres of *H. bulbosum* carry less CENH3 protein and showing therefore, weaker CENH3-signals (Fig. 7A arrowed). No CENH3-signals were detected in micronuclei (Fig. 7B). Therefore, the centromere of micronucleated chromatin is not functional and will undergo elimination. Absence of CENH3 protein in lagging chromosomes, weak CENH3 signal in *H. bulbosum* centromeres and lack of CENH3 in micronuclei indicate that CENH3 in the *H. bulbosum* chromosomes dilute during cell cycles and is not reloaded with new CENH3 protein, therefore it cause a defect in the centromere activity and subsequent elimination of the *H. bulbosum* chromosomes. Overall it shows that the CENH3 has a potential role in the process of uniparental chromosome elimination.

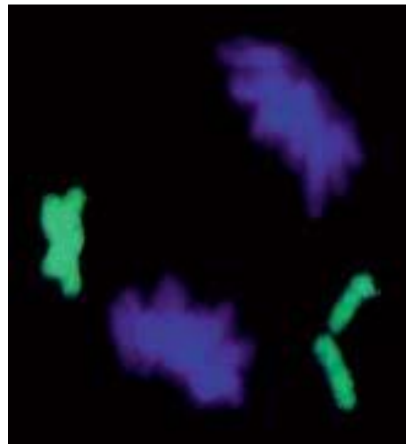


Fig. 6. Anaphase cells of *H. vulgare* x *H. bulbosum* embryos after *in situ* hybridization with genomic DNA of *H. bulbosum* (in green). Chromosomes of *H. vulgare* are shown in blue (Gernand et al., 2006).

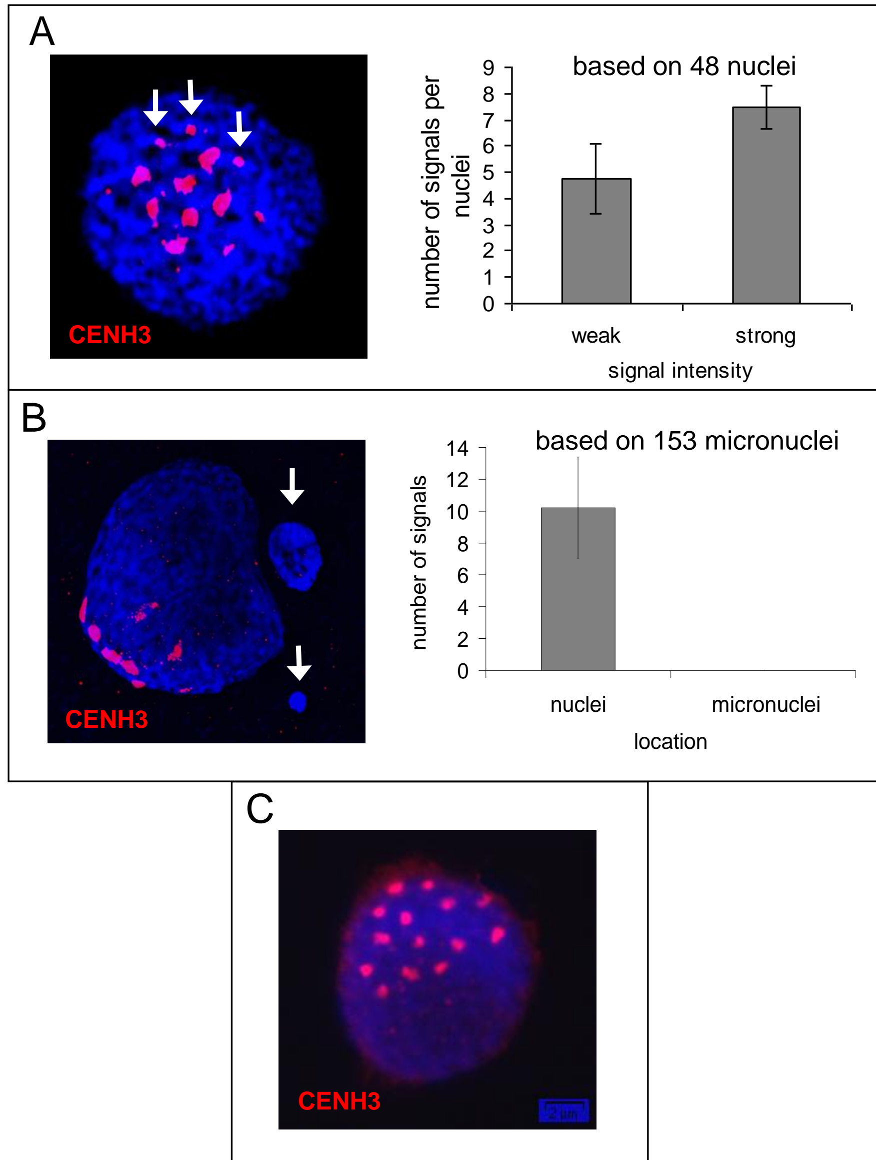


Fig. 7. *H. bulbosum* centromeres show weaker CENH3 signals in (A) some nuclei (arrowed) and (B) no signal in micronuclei (arrowed) of unstable hybrid embryos. Histogram in (A) shows number of weak and strong signals per nuclei, and (B) shows number of signals per nuclei and micronuclei (±SD). (C) Stable hybrid nuclei show 14 signals with similar intensity as control.

4.3. Identification of *H. vulgare*, *H. bulbosum* and *H. marinum* CENH3 genes

Lack of detectable CENH3 protein in *H. bulbosum* centromeres indicate an active role of CENH3 in the elimination process of *H. bulbosum* chromosomes.

Therefore, it was assumed that the CENH3 gene of *H. bulbo*sum undergoes silencing in unstable hybrid. To test this hypothesis, CENH3s of the *Hordeum* species were cloned, sequenced and the expression of parental CENH3s in stable and unstable hybrid embryos were examined.

Fig. 8. A schematic illustration of primer positions that were used for amplification of the CENH3s of *H. vulgare*, *H. bulbosum* and *H. marinum*.

4.3.1. Isolation of *H. vulgare* CENH3s

CENH3 of *H. vulgare* was amplified using a previously published grass-specific CENH3 primer (primers 5 and 12) (Nagaki et al., 2005a). RT-PCR amplified two variants of CENH3 with a length of 251 bp and 273 bp. The two variants of *H. vulgare* CENH3 were called HvαCENH3 and HvβCENH3. Blast analysis of the Genebank database resulted the identification of the 5' region of HvαCENH3 (GenBank BU996921) and HvβCENH3 (GenBank AK249602). 3' UTR of the HvβCENH3 was amplified by Race PCR using primer 4 (resulting in a 588 bp fragment) (Fig. 8).

4.3.2. Isolation of *H. bulbosum* and *H. marinum* CENH3s

From the sequence of HvαCENH3, a primer pair (primers 1 and 3) was deduced to amplify αCENH3s of *H. bulbosum* and *H. marinum*. The two variants of *H. bulbosum* CENH3 were called HbαCENH3 and HbβCENH3. The two CENH3s of *H. marinum* were called HmαCENH3 and HmβCENH3. RT-PCR resulted in the amplification of 225 bp and 204 bp long fragments for *H. bulbosum* and *H. marinum,* respectively. The neighbouring regions were amplified with primers 5 and 12 resulted a 252 bp fragment from *H. bulbosum.* Primer 4 and 11 (deduced from HvαCENH3) resulted a 221 bp fragment from *H. marinum* (Fig. 8). HbβCENH3 was amplified as a result of Race PCR (using primer 4 on *H. bulbosum*), and extended using HvβCENH3-specific primers (primers 2 and 13). HmβCENH3 were amplified using primer 4 and 13 (deduced from HvβCENH3) (Fig. 8).

As a result, we isolated two variants of *H. vulgare* CENH3 (called HvαCENH3, Genbank number, JF419328 and HvβCENH3, Genebank number, JF419329), two variants of *H. bulbosum* CENH3 (HbαCENH3, Genbank number, GU245882.1 and HbβCENH3, Genbank number, JF419330) and two variants of *H. marinum* CENH3 (HmαCENH3, Genbank number, GU245883.1 and HmβCENH3, Genbank number, JF720032) (Appendix Fig.1) A pair-wise comparison of amino acid identity and similarity of *H. vulgare*, *H. bulbosum* and *H. marinum* CENH3s is shown in appendix table 1.

The deduced amino acid sequences of CENH3 were compared with sequences encoding CENH3 of maize (accession number AF519807; (Zhong et al., 2002), rice (accession number AY438639; (Nagaki et al., 2004), sugar cane (The Institute for Genomic Research EST sequences CA127217 and TC19006; (Kato et al., 2006; Nagaki and Murata, 2005) and of *Luzula nivea* (accession number AB201356; (Nagaki et al., 2005a)) to determine conserved regions (Fig. 9).

The conserved αN-helix, α1-helix, α2-helix, α3-helix and loop 1 regions (Vermaak et al., 2002) of CENH3 were determined (Fig. 9). The N-terminal ends were highly variable but the first five amino residues were completely conserved among the CENH3s listed. βCENH3 types are shorter in N-terminal region in comparison with αCENH3 types and longer in loop1 region. Difference in the loop1 regions of α and β CENH3 types of each species indicates their possible different targeting site on centromeric DNA (Vermaak et al., 2002).

```
                    *       20        *       40        *       60        *       80        *
OsCENH3  : MARTKHPAVRKSKAEPKKKLQFERSPRPSKAQRAGGGTG-TSATTR----SAAGTSASGTPR--QQTKQRKPHRFRPGTVALREIRKFQK :  83
ZmCENH3  : MARTKHQAVRKTAEKPKKKLQFERS----------GGAS-TSATPE----RAAGTGGRAASGGDSVKKTKPRHRWRPGTVALREIRKYQK :  75
LnCENH3  : MARTKHFPQCSRHPKKQRTAAGEAGSSVIAKQNAPAKTGNASSITNSTPARSLKKNKASKRGEKTQAKQRKMYRYRPGTVALREIRKLQK :  90
SoCENH3  : MARTKHQAVRRPTQKPKKKLQFERA----------GGAS-TSATPE----RNAGTGGGAAAR-VTRGRVEKKLRWRAGTVALREIRKYQK :  74
HvαCENH3 : MARTKHPAVRKSKAPPKKKIGSASSPSAAQRRQETDGAG-TSETPRRAGRGPAPAAAEGAPG--EPTK-RKPHRFRPGTVALREIRKYQK :  86
HbαCENH3 : MARTKHPAVRKSKAPPRKKVGSARAPAAAQRRHETDGAG-TSETPR---RGPAPAADQGAPG--EPKK-RKPHRYRPGTVALREIRKYQK :  83
HmαCENH3 : MARTKHQAVRKTAEKPKKKLQFERS----------GGAS-TSATPE----RAAGTGGRAASGGDSVKKTKPRHRWRPGTVALREIRKYQK :  75
HvßCENH3 : MARTKKTVAAKEKRPPCSKSEPQSQ-------------------PK--------------------KKEKRAYRFRPGTVALREIRKYRK :  51
HbßCENH3 : MARTKKTVAATKRRSPRTRLEPQSQ-------------------PE--------------------KK-KRAHRFRPGTVALREIRKYRK :  50
HmßCENH3 : ---------------------------------------------------------------------------VALREIRKYQK :  11
                                                                                       αN-helix

                              CATD
                100       *       120       *       140       *       160       *
OsCENH3  : TTELLIPFAPFSRLVREITDFYSK-------DVSRWTLEALLALQEAAEYHLVDIFEVSNLCAIHAKRVTIMQKDMQLARRIGGRRPW : 164
ZmCENH3  : STEPLIPFAPFVRVVRELTNFVTNG------KVERYTAEALLALQEAAEFHLIELFEMANLCAIHAKRVTIMQKDIQLARRIGGRRWA : 157
LnCENH3  : TTDLLVPKASFARLVKEITFQSSK-------EVNRWQAEALIALQEASECFLVNLLESANMLAIHARRVTIMKKDIQLARRIGA---- : 167
SoCENH3  : STEPLIPFAPFVRVVKELTGFITDW------RIGRYTPEALLALQEAAEFHLIELFEVANLCAIHAKRVTVMQKDIQLARRIGGKRW- : 155
HvαCENH3 : SVNFLIPFAPFVRLVREITEYYCP-------RVKRWTPQALLAVQEATEYHLVDIFERAHLCAIHAKRVTVMQKDMQLAR-------- : 159
HbαCENH3 : SVDFLIPFAPFVRLVKEVTEFYCP-------AISRWTPQALLAVQEAAEYHLVDVFERAHLCAIHAKRVTVMQKDIQLAR-------- : 156
HmαCENH3 : STEPLIPFAPFVRVVRELTNFVTNG------KVERYTAEALLALQEAAEFHLIELFEMANLCAIHAKRVTIMQKDIQLARRIGGRRWA : 157
HvßCENH3 : STNMLIPFAPFVRLVRDIADNLTPLSNKKESKPTPWTPLALLSLQESAEYHLVDLFGKANLCAIHSHRVTIMLKDMQLARRIGTRSLW : 139
HbßCENH3 : STELLIPFAPFVRLVRGIANGFMNIGG--PNKPTPWTPHALLSLQEAAEYHLVDLFGKANLCAIHAKRVTVLLKDMRLAKRIGSVTV- : 135
HmßCENH3 : STGLLIPFAPFVRLVKEITNDLT------KGELNHWTPQALVSLQEAAEYHIVDLFEKANLCAIHAKRVTMMQKDIQLARRIGGTRLW :  93
                  α1-helix              Loop1          α2-helix              Loop2  α2-helix
```

Fig. 9. Amino acid sequence alignment of CENH3s of *Hordeum* species with those of other grass CENH3s. Alignment includes CENH3 of rice (OsCENH3), maize (ZmCENH3), *Luzula nivea* (LnCENH3), sugar cane (SoCENH3), αCENH3-type of *H. vulgare* (HvαCENH3), *H. bulbosum* (HbαCENH3) and *H. marinum* (HmαCENH3) and βCENH3-type of *H. vulgare* (HvβCENH3), *H. bulbosum* (HbβCENH3) and *H. marinum* (HmβCENH3). Well conserved histone-folding domains similar to that of other eukaryotes (Henikoff et al., 2001) are indicated.

Phylogenetic analysis of the amino acid sequence of the CENH3s of *Hordeum* species with the CENH3s of some other species (Fig 10) shows that the αCENH3s and βCENH3s of *Hordeum* species form two distinct subclusters. Therefore, it is likely that the members of each subcluster evolved from the same origin. In addition, *Hordeum* αCENH3s cluster with the CENH3s of rice, maize and sugar cane. Indicating that the αCENH3-type is the evolutionarily older

CENH3 variant and βCENH3s likely originated via duplication of CENH3. BLAST analysis of the rice genome did not allow the identification of a βCENH3-type.

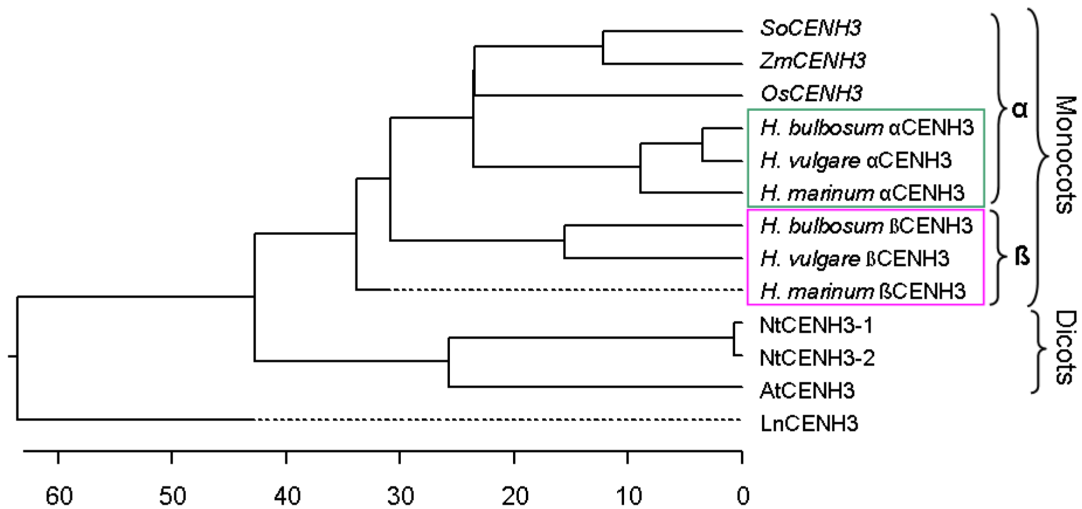


Fig. 10. The phylogenetic tree of CENH3s amino acids of rice (OsCENH3), maize (ZmCENH3), *Luzula nivea* (LnCENH3), sugar cane (SoCENH3), αCENH3-type of *H. vulgare* (HvαCENH3), *H. bulbosum* (HbαCENH3) and *H. marinum* (HmαCENH3) and βCENH3-type of *H. vulgare* (HvβCENH3), *H. bulbosum* (HbβCENH3) and *H. marinum* (HmβCENH3). Clustering of αCENH3 with the CENH3s of other grasses indicates that the αCENH3 type is the evolutionarily older CENH3-type that underwent duplication in the genus *Hordeum.* Phylogenetic tree was generated by the Neighbour Joining method.

4.3.3. Intron/exon structure of *Hordeum* CENH3 genes

For further characterization of the CENH3 genes of *H. vulgare* and *H. bulbosum*, the intron/exon structure was analysed. Based on the intron/exon structure of rice CENH3, different primer pairs were selected and applied to amplify parts of the genomic CENH3 regions of *H. vulgare* and *H. bulbosum*. The intron/exon junctions were identified via comparison of genomic DNA with corresponding cDNA. This was done by Martinez-MW method in MegAlign application of Lasergene software. Comparing the gene structure of CENH3s shows that the splicing sites are conserved across CENH3 of rice and of αCENH3s of *H. vulgare* and *H. bulbosum* (Fig. 11).

As the PCR amplification of genomic *Hordeum* βCENH3s was less successful, it indicated that the βCENH3 has probably a different gene structure (Fig. 11).
Exons 1, 2, 3, 4 and 5 of HvαCENH3, exons 1, 2, 3 and 4 of HbαCENH3, exons 4 and 5 of HvβCENH3 and 4 and 5 of HbβCENH3 were amplified by PCR.

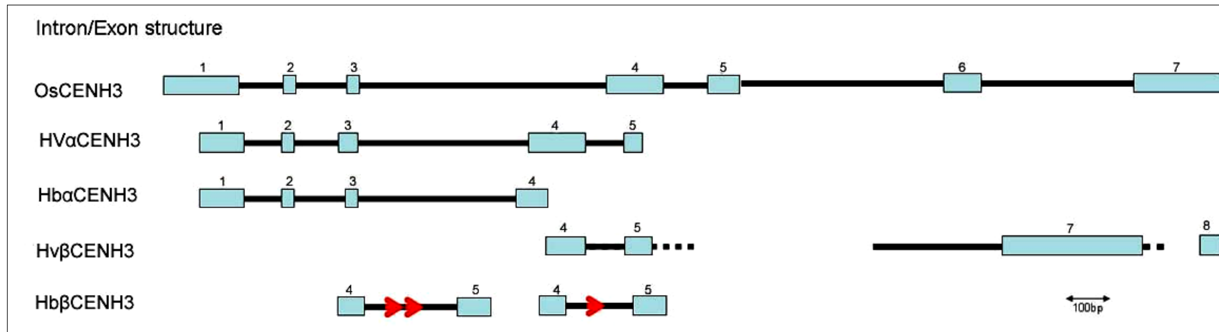


Fig. 11. Gene model of the CENH3s of rice, *H. vulgare* and *H. bulbosum*. Exons are represented as boxes and introns as lines. Arrows show duplicated region in an intron of HbβCENH3.
Note, the genomic DNA of *Hordeum* CENH3s is incomplete.

One other exon (exon 7) and intron of 3' region of HvβCENH3 (accession F151T9F01BD00B) were identified by BLAST searching (http://pgrc.ipk-gatersleben.de/blast/barlex/blast.cgi) in internal IPK database (454 whole genome shotgun sequence of the barley cultivar 'Betzes'). Table 4. shows the origin and features of the genomic sequences that were amplified.

Table 4. Features of the genomic sequences of HvαCENH3, HbαCENH3, HvβCENH3 and HbβCENH3.

Gene	Origin	Region	Fragment size
HvαCENH3	PCR product of primers1 and 10	N-terminal and a part of C-terminal region (exons 1, 2, 3, 4 and 5)	977 bp
HbαCENH3	PCR product of primers 1 and 3	N-terminal and a part of C-terminal region (exons 1, 2, 3 and 4)	789 bp
HvβCENH3	PCR product of primers 4 and 8	Part of C-terminal region (CENH3 targeting domain, exons 4 and 5)	332 bp
	Data base(F151T9F01BD00B)	Part of 3' UTR (exons 7 and 8)	518 bp
HbβCENH3	PCR product of primers 4 and 7	Part of C-terminal region (CENH3 targeting domain, exons 4 and 5)	284 and 331 bp

Primers specific for the N-terminal region of βCENH3s based on intron/exon structure of OsCENH3, did not amplify any genomic sequence of βCENH3s. In addition, one intron is inserted in 7th exon of HvβCENH3. Overall we can conclude that βCENH3 has a slightly different gene organization. But it is conserved in exons 4 and 5 region that encodes the active site of the CENH3 gene (loop1 locates on exon 5); while gene structure of αCENH3 and OsCENH3 are identical (Fig. 11).

A duplication of a 47 bp fragment within the forth intron of HbβCENH3 was discovered in different *H. bulbosum* genotypes (Appendix Fig. 2 and Fig. 11). Genotype 2920/4 has two alleles of HbβCENH3 (one allele with a duplication and other without). The genotypes 2929/1, 3811/3, A14 and 2023 posses only the allele with the duplicated intron 4 (Fig. 12). It is unlikely that the duplication results in a significant effect on the structure or level of CENH3 mRNA as the 5' and 3' splicing sequences are unaffected by the duplication (Appendix Fig. 2 and 11). Splicing sites are identical for αCENH3s of *Hordeum* and OsCENH3. *Hordeum* βCENH3s are characterized by a small shift in exons 4 and 5.

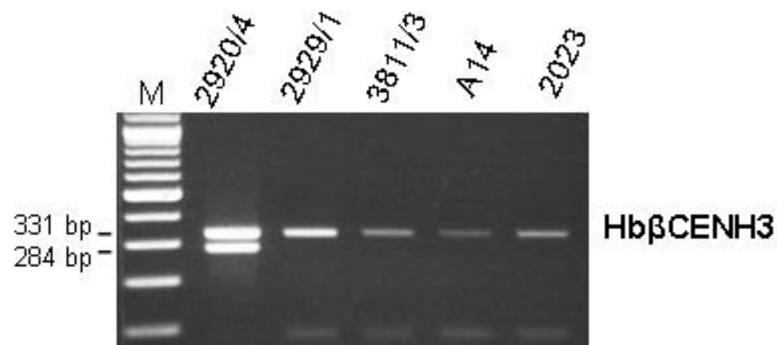


Fig. 12. Genomic PCR analysis using primers 4 and 7on different *H. bulbosum* genotypes (2920/4, 2929/1, 3811/3, A14 and 2023), genotype 2920/4 is heterozygous for HbβCENH3.

4.4. Determination of the chromosomal location of CENH3 genes

4.4.1. Mapping of the barley CENH3 genes

Seven wheat-barley addition lines were utilized to determine which barley chromosome carries CENH3 genes. Each disomic addition line possesses the full complement of wheat chromosomes including barley chromosome 2H, 3H, 4H, 5H, 6H or 7H. A stable and self-maintaining wheat-barley disomic addition line for chromosome 1H is not available due to a gene on 1HL causing sterility when present in wheat (Taketa et al., 2002). Instead, a double disomic addition for 1H + 6H was used. The plant material was provided by A.K.M.R. Islam (Adelaide, Australia),

For localization of HvαCENH3, primer 6 and 16 were used on the genomic DNA of all available wheat-barley addition lines. Barley and wheat genomic DNA were used as positive and negative control. A HvαCENH3-specific product was obtained only for the wheat-barley double disomic addition of 1H + 6H, but not for the 6H addition line. Hence, HvαCENH3 locates on chromosome 1H (Fig. 13).

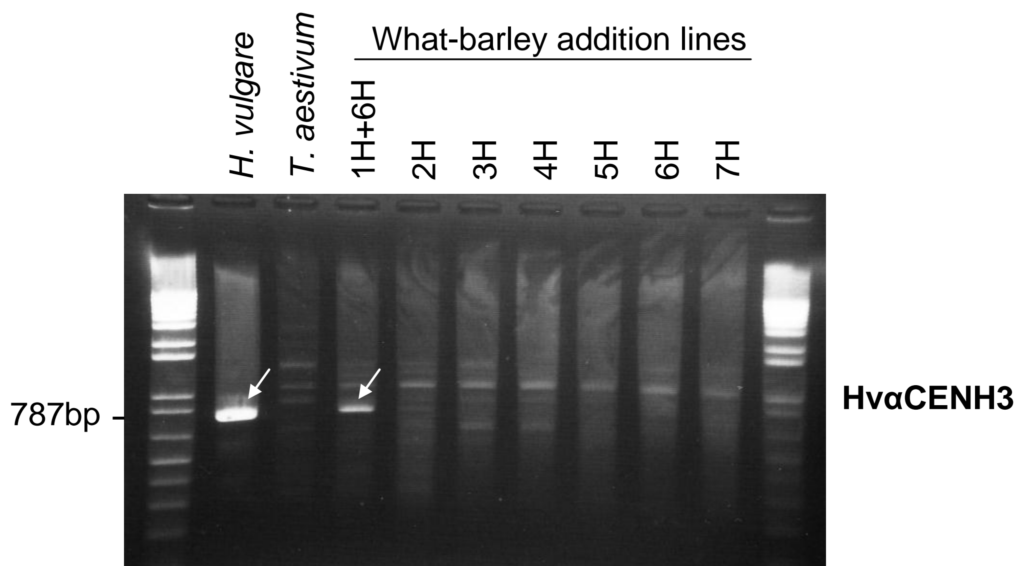


Fig. 13. Determination of the chromosomal location of HvαCENH3, using HvαCENH3 specific primers on gDNA of different wheat-barley addition lines. Genomic DNA of barley and wheat was used as positive and negative controls. Arrows indicate HvαCENH3-specific product. Result shows that the HvαCENH3 locates on chromosome 1H of barley.

PCR using genomic DNA of the different wheat-barley addition lines and the primer 4 and 8 allowed the localization of HvβCENH3 on chromosome 6H (Fig. 14). Barley (cultivar Betzes) and wheat genomic DNA were used as positive and negative control. HvβCENH3-specific products were obtained for the wheat-barley addition lines 6H and 1H+6H indicates that the HvβCENH3 locates on chromosome 6H of barley. The mapping experiment was performed in collaboration with H. Nasuda (Kyoto, Japan).

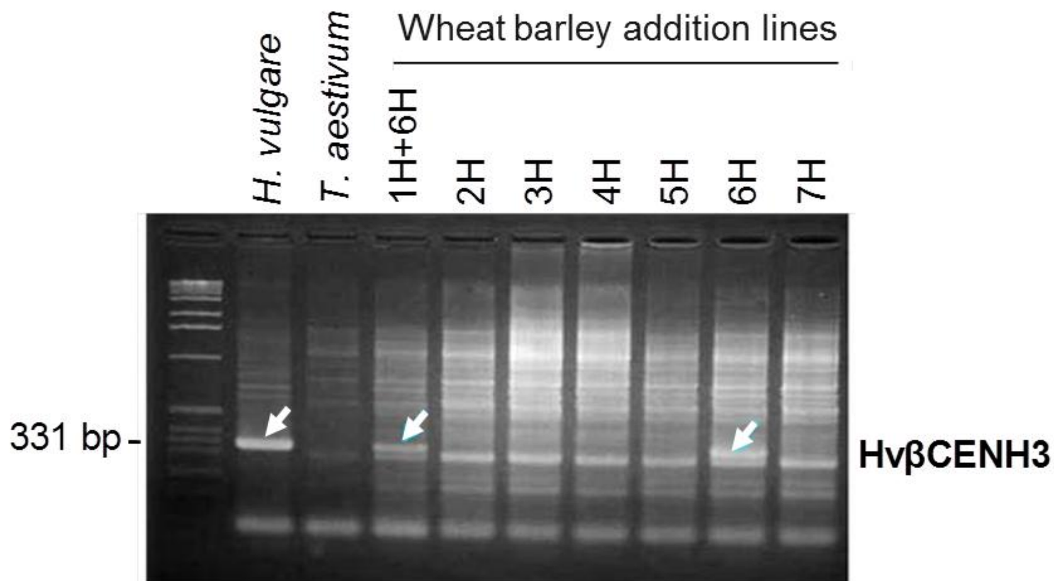


Fig. 14. Determination of the chromosomal location of HvβCENH3, using HvβCENH3 specific primers on genomic DNA of wheat-barley addition lines. Barley and Wheat genomic DNA were used as positive and negative control. Arrows indicate a HvβCENH3-specific product. Result shows that this gene locates on chromosome 6H of barley.

4.4.2. Mapping of *H. bulbosum* CENH3 genes

For mapping of the *H. bulbosum* CENH3 genes we applied different *H. vulgare*/*H. bulbosum* introgression lines and a number of *H. vulgare/H. bulbosum* substitution lines. The *H. bulbosum*-specific primers 17 and 18 were used for genomic PCR. No substitution line for 1H of *H. bulbosum* is available thus we used indirect proving as 6 other different barley line with substitution of all other *H. bulbosum* chromosomes were negative for HbαCENH3 gene (Fig. 15).

Therefore, we conclude that HbαCENH3 is located on chromosome 1H. For the mapping experiment four *H. vulgare* cultivars (parents of the substitution lines were used as negative control. DNA of *H. bulbosum* (2920/4) was used as positive control (Fig. 15). A primer pair specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for control PCR experiments (primers 19 and 20).

In addition, we used three lines (333Y1, 315A1, 230H1/7M1/M1) with introgressions of distal parts of *H. bulbosum* 1H chromosome into the *H. vulgare* genome (Table 2). Result shows that HbαCENH3 gene is not located on these parts of 1H of *H. bulbosum* (Fig. 16). DNA of *H. vulgare* and of 6H introgression line were used as negative control. Genomic DNA of *H. bulbosum* was used as positive control. The plant material was provided by R. Pickering (Christchurch, New Zealand).

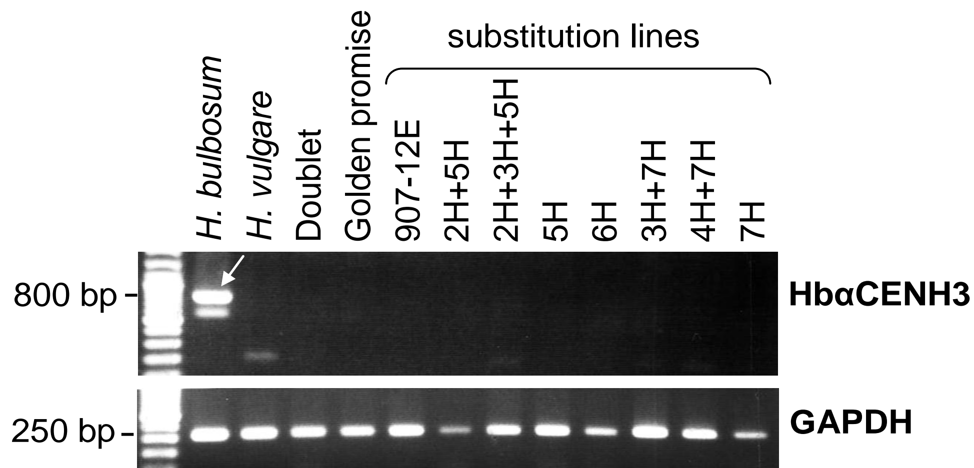


Fig. 15. Amplification of HbαCENH3 in *H. bulbosum*, but not in *H. vulgare* genome substituted with different chromosomes of *H. bulbosum* (1H substitution line is not available), indirectly indicates that the HbαCENH3 is located on chromosome 1H of *H. bulbosum.* Emir, Doublet, Golden promise and 907-12E are different barley cultivars that were used as a parent for generation of substitution lines and here were used as negative control; *H. bulbosum* gDNA was used as positive control. GAPDH primers were used as positive control.

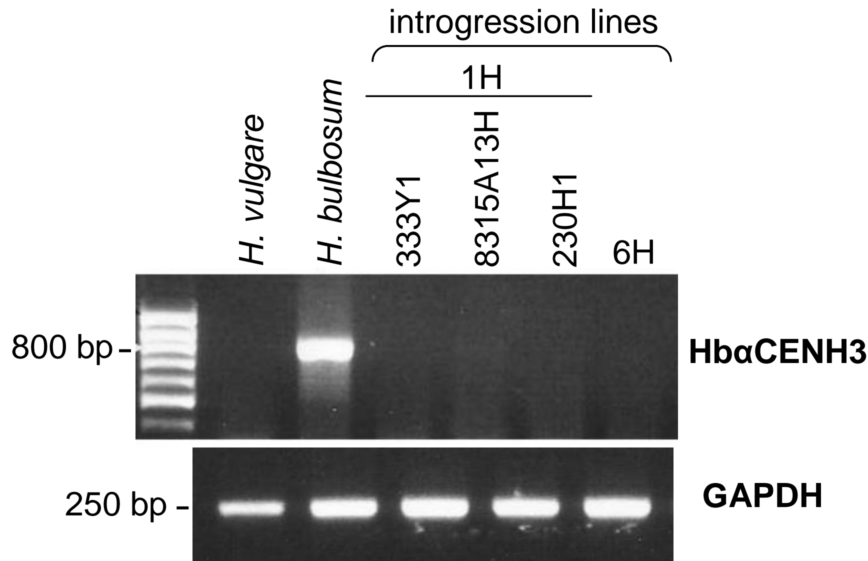


Fig. 16. Using three lines with introgressions of some parts of chromosome 1H of *H. bulbosum* to the *H. vulgare* genome for further localization of the HbαCENH3 gene. GAPDH was used as positive control. Result shows that the HbαCENH3 is not located on distal part of the 1H chromosome of *H. bulbosum*.

Five *H. vulgare*/*H.bulbosum* 6H substitution lines (919Q4, 16R5, 38M1, 38M11 and 36L46) and an introgression line (916J2/2) with introgression of 6HL (half of the arm) and small distal introgression on the 6HS of *H. bulbosum* to the barley genome were used to determine whether HbβCENH3 locates on chromosome 6H. This was assumed as HvβCENH3 locates on 6H of *H. vulgare*. Primers 4 and 7 were used on the genomic DNA for PCR. HbβCENH3--specific products were obtained from *H. vulgare*/*H. bulbosum* 6H substitution lines indicates that HbβCENH3 locates on 6H of *H. bulbosum*. No amplification from the introgression line 916J2/2 indicates that HbβCENH3 gene locates in an interstitial region of chromosome 6H (Fig. 17).

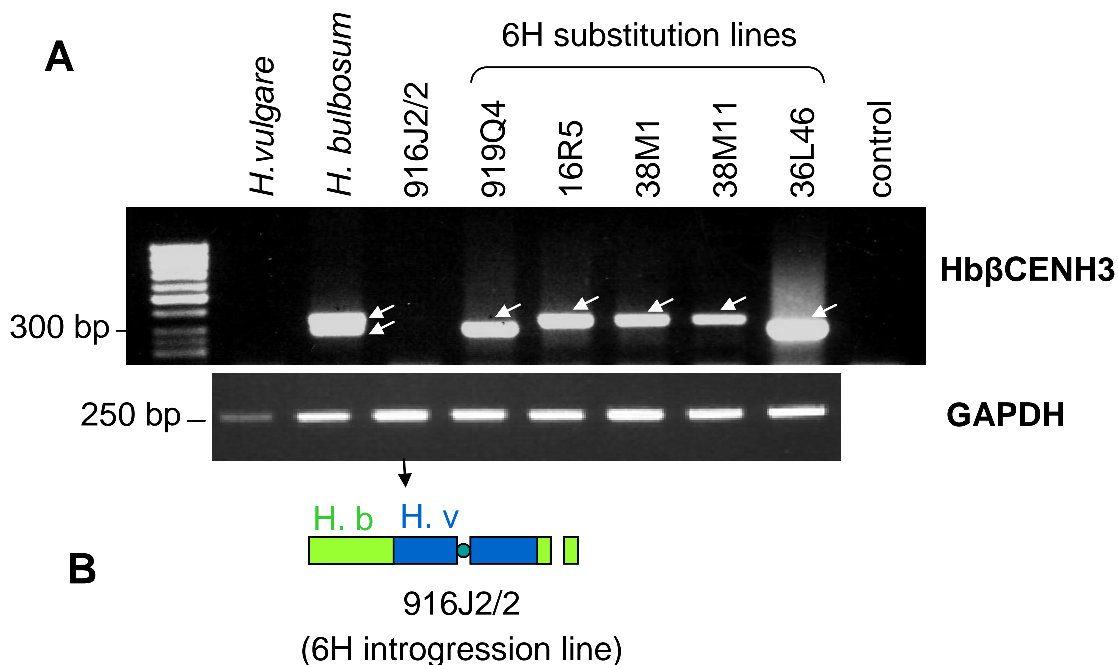


Fig. 17. Determination of the chromosomal location of HbβCENH3, (A) using a 6H *H. bulbosum* introgression line (with a large introgression) and different *H. vulgare*/*H.bulbosum* 6H substitution lines (Table 2). Result shows that this gene is located in a distal region of chromosome 6 of *H. bulbosum.* Arrows show two alleles of the HvβCENH3 in *H. bulbosum* parent which one of them was detected in substitution lines (as only one 6H chromosome of *H. bulbosum* were substituted with 6H of barley). (B) Feature of introgression line 916J2/2 as schemata.

4.5. Testing the expression of parental CENH3 genes in stable and unstable *H. bulbosum* x *H. vulgare* hybrids

Chromosome elimination is strongly influenced by the genotype of the parents used for species combination. *H. bulbosum* genotype 2920/4 in combination with *H. vulgare* (cv Emir) causes a high chromosome elimination rate in hybrid embryos. In contrast, *H. bulbosum* genotype 3811/3 in combination with Emir causes a high rate of chromosome retention .In addition; the temperature during the early stages of embryo formation is influencing the rate of chromosome elimination. A temperature <18°C is conducive for chromosome retention, above 18°C the rate of chromosome elimination is increased (Pickering, 1983). Highest rate of chromosome elimination occurs in cross of *H. vulgare* (cv Emir) x *H.*

bulbosum (2920/4) in warm condition that is around 94%, and highest rate of chromosome retention occurs in cross of *H. vulgare* (cv Emir) x *H. bulbosum* (3811/3) in cool condition that is 100% (Pickering, 1983, 1984).

As lagging chromosomes of *H. bulbosum* did not display CENH3-specific immunosignals (Fig. 5A), we assumed that inactivation of the HbCENH3 genes occurs, and CENH3s of *H. vulgare* does not compensate the lacking CENH3 of *H. bulbosum*.

To test whether parent-specific inactivation of CENH3 happens the expression activity of parental CENH3s was tested in young hybrid embryos. Therefore, stable and unstable hybrid embryos of different ages (5 - 11 days after pollination) were microisolated and 2 - 5 embryos of each age and type were pooled. After RNA isolation and cDNA synthesis the transcription activity of parental CENH3s was investigated by RT-PCR. For the detection of CENH3 transcripts we used α or β CENH3-type-specific primer pairs (one primer pair for αCENH3, and one primer pair for βCENH3 of both species). To distinguish the parental origin, the PCR products were cleaved with an enzyme which cuts at a *H. bulbosum*-specific restriction site (CAPS analysis). Stable hybrid embryos (*H. vulgare* (cv Emir) x *H. bulbosum* (3811/3), cool condition) were included as a positive control for the transcription of CENH3s of both parents (Fig. 20).

The CAPS analysis was performed first on both barley species separately as a control. RT-PCR amplification with primers specific for the transcripts of αCENH3 of both species (primers 17 and 18) resulted in the amplification of HvαCENH3 and HbαCENH3. Incubation with *Alw*I cleaved the *H. bulbosum* derived PCR products into two fragments of 210 bp and 54 bp, but left the *H. vulgare* derived product uncleaved (Fig. 18A).

Similarly, RT-PCR amplification with primers specific for the transcript of βCENH3 of both species (primer 2 and 13) resulted in HvβCENH3 and

HbβCENH3 amplification. Incubation with *Ban*II cleaved the *H. bulbosum* derived PCR products into two fragments of 398 bp and 234 bp, but left the *H. vulgare* derived product uncleaved (Fig. 18B).

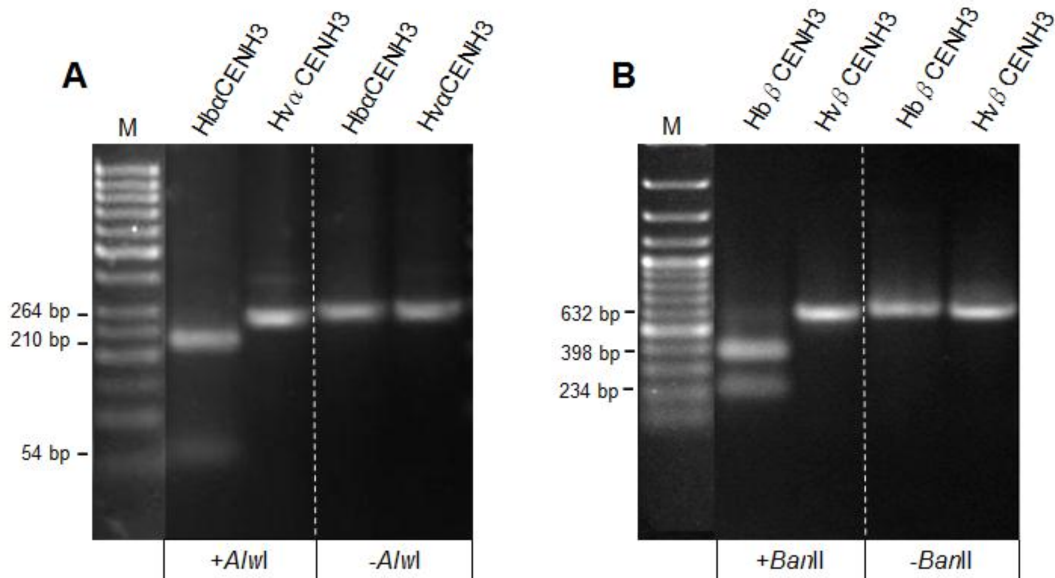


Fig. 18. Test CAPS analysis for the detection of parental CENH3 transcripts using RT-PCR products derived from parental cDNA. (A) αCENH3s of *H. bulbosum* and *H. vulgare* are distinguishable by *Alw*I digestion (+*Alw*I). (B) βCENH3s of *H. bulbosum* and *H. vulgare* are distinguishable by *Ban*II digestion (+*Ban*II). Controls (-*Alw*I and -*Ban*II) are undigested PCR products.

4.5.1. CENH3 genes of both parental genomes are equally active in chromosomally stable and unstable hybrids

To test whether CENH3s of both parental genomes are active in stable and unstable hybrids we assayed their corresponding transcription. For that parental CENH3 transcription in stable hybrid embryos from cross of *H. vulgare* (cv Emir) x *H. bulbosum* (3811/3) in cool environment and unstable hybrid embryos from cross of *H. vulgare* (cv Emir) x *H. bulbosum* (2920/4) in warm environment were compared. Over all around 250 hybrid embryos were tested.

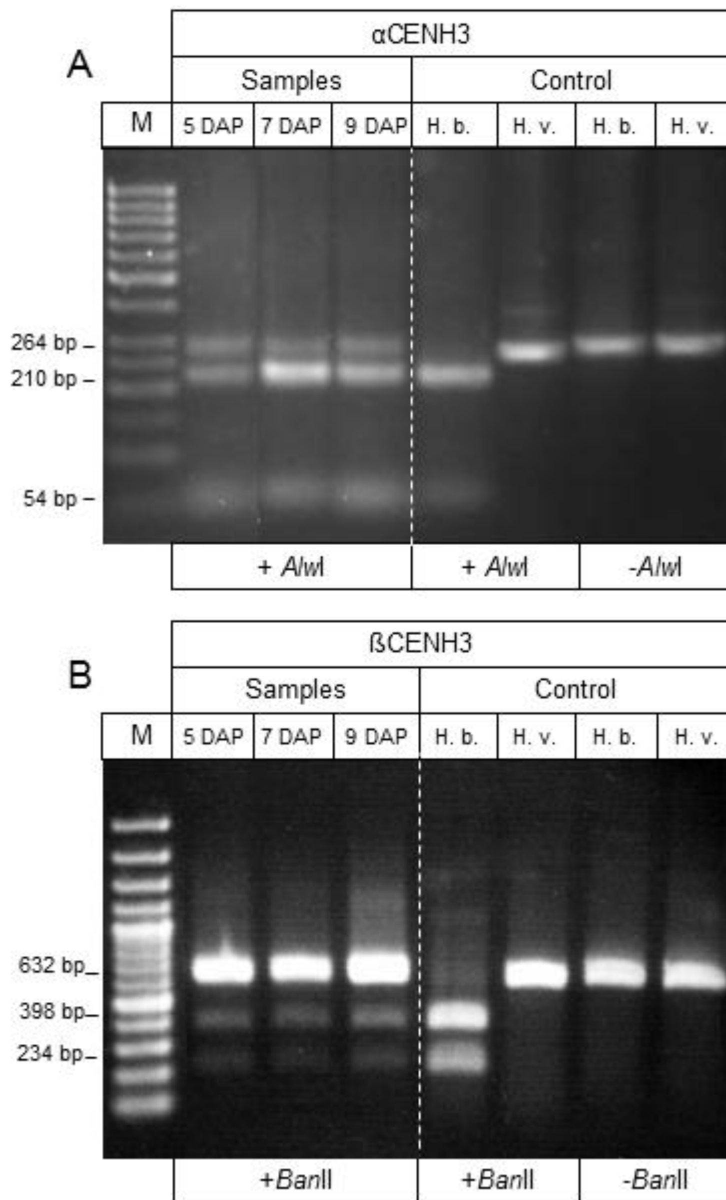


Fig. 19. Expression analysis of parental CENH3s in unstable hybrid (*H. vulgare* x *H. bulbosum*) embryos undergoing chromosome elimination. Embryos were isolated 5, 7 and 9 days after pollination (DAP). Cleaved fragments correspond to HbCENH3 transcripts, and uncleaved fragments show transcripts of HvCENH3. (A) Expression analysis of HvαCENH3 and HbαCENH3 and (B) analysis of HvβCENH3 and HbβCENH3. CAPS analysis of parental CENH3s (*H. bulbosum* (H.b.) and *H. vulgare* (H.v.)) was used as control. Analysis shows that all parental CENH3s express in unstable hybrid embryos of different ages.

CAPS analysis of cDNA from *H. vulgare* x *H. bulbosum* hybrids resulted after *Ban*II and *Alw*I cleavage, in fragments corresponding in size to transcripts derived from four CENH3s of both parental genomes in stable hybrid embryos (Fig. 20). The same was found for unstable hybrid embryos (Fig. 19). The expression patterns were similar independent of the embryo age (Fig. 19). Indicating that the expected inactivation of the HbCENH3 genes does not occur in unstable hybrids. Therefore, CENH3 gene inactivity is not responsible for the missing CENH3 proteins of *H. bulbosum* chromosomes undergoing elimination.

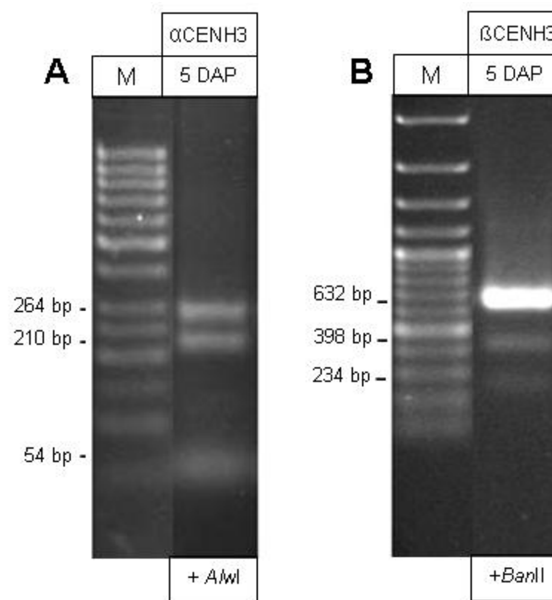


Fig. 20. Expression analysis of parental CENH3s in stable hybrid embryos of *H. vulgare* x *H. bulbosum*. Embryos were isolated 5 days after pollination (DAP). Cleaved fragments correspond to HbCENH3 transcripts, and uncleaved fragments show transcripts of HvCENH3. (A) Expression analysis of HvαCENH3 and HbαCENH3 and (B) analysis of HvβCENH3 and HbβCENH3.

To investigate whether the differences in intensity of the cleaved βCENH3 fragments (HbβCENH3) and uncleaved fragments (HvβCENH3) after digestion with *Ban*II are due to different expression level of parental βCENH3s in hybrid embryo or because of technical reason, a test PCR was performed on cloned HvβCENH3 and HbβCENH3 cDNA. Therefore, equal amounts of recombinant plasmid DNA (cloned HvβCENH3 and cloned HbβCENH3) were mixed in equal proportions and used as template for amplification with primers 2 and 13. After

PCR the products were cleaved with *Ban*II and gel separated. Plasmid DNA of cloned HvβCENH3 or HbβCENH3 was used as control. Figure 22 shows that although equal amounts of HvβCENH3 and HbβCENH3 as PCR template were used, after *Ban*II digestion the resulting DNA fragments were of different amount. Therefore, it is likely that the βCENH3-specific primer pair binds preferentially to HvβCENH3 transcripts and the observed difference in figure 19B does not indicate a differential expression of parental βCENH3 in hybrid embryos.

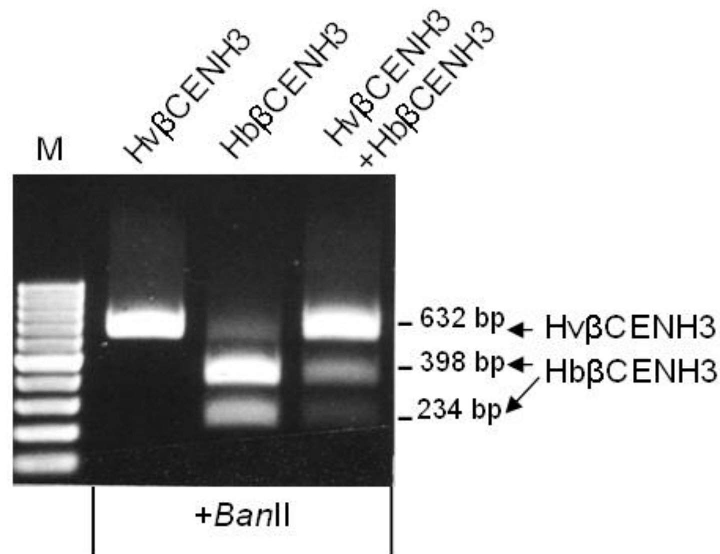


Fig. 21. Comparing quantity of digested and undigested fragment (arrows) after amplification of a mixture of same amount of HvβCENH3 and HbβCENH3 DNA. Result shows different intensity of cleaved and uncleavd band although starting material has equal proportion. Indicating that observed difference intensity of HvβCENH3 and HbβCENH3 in hybrid embryo is due to technical reasons.

As post-pollination temperature has an effect on the process of chromosome elimination (warm temperature (>18°C) favors chromosome elimination, whereas cool temperature (<18°C) results in a higher proportions of hybrids) (Pickering, 1984). Parental CENH3 transcription was examined in order to investigate whether temperature influences chromosome elimination via change in CENH3 transcription pattern. Young hybrid embryos (5, 7, 9, and 11 DAP, *H. vulgare* (cv Emir) pollinated with *H. bulbosum* (3811/3)) were isolated at two contrasting temperatures (12°C and 21.5°C) and investigated for the parental CENH3s

expression. Result shows that α and β CENH3s of *H. vulgare* similar to α and βCENH3 of *H. bulbosum* undergoes transcription in warm and cool environment similarly (Table 5). This result again indicates that silencing of HbCENH3 genes is not the cause of chromosome elimination in hybrids.

Table 5. Summary of the transcription patterns of parental CENH3s in different cross combinations. *H. vulgare* (cv Emir) was used as female and *H. bulbosum* (genotype 2920/4 or 3811/3) as pollinator. Results show that all parental CENH3 genes similarly express in unstable and stable hybrids, independent to the temperature or the parent genotype.

Cross combination	Condition and hybrid stability	Parental genes	5 DAP	7 DAP	9 DAP	11 DAP
Emir x 2920/4	Warm (unstable hybrid)	HvαCENH3 HvβCENH3 HbαCENH3 HbβCENH3	+ + + +	+ + + +	+ + + +	+ + + +
Emir x 3811/3	Cool (stable hybrid)	HvαCENH3 HvβCENH3 HbαCENH3 HbβCENH3	+ + + +	+ + + +	+ + + +	+ + + +
Emir x 3811/3	Warm (stable hybrid)	HvαCENH3 HvβCENH3 HbαCENH3 HbβCENH3	+ + + +	+ + + +	+ + + +	+ + + +

4.6. Characterization of αCENH3 and βCENH3 proteins of *H. vulgare*

4.6.1. Generation and characterization of *H. vulgare* α and βCENH3-specific antibodies

The existence of a second CENH3 variant in a diploid monocot, like barley was surprising. Known diploid organisms with multiple CENH3s are *Arabidopsis halleri* and *A. lyrata* (Kawabe et al., 2006), *Caenorhabditis elegans* (Monen et al., 2005) and cow (Li and Huang, 2008). However, in all mentioned cases the function of the duplicated CENH3 gene was not studied, yet.

In order to characterize the function and chromosomal distribution of multiple CENH3s, specific antibodies against HvαCENH3 and HvβCENH3 were generated. For the *in situ* detection of *H. vulgare* α and βCENH3 proteins, two polyclonal peptide antibodies were raised. Suitable CENH3-type-specific epitopes were identified (CQRRQETDGAGTSETPRRAGR and CAEGAPGEPTKRKPHRFR for generating a HvαCENH3--specific antibody, a double peptide antibody, and CSKSEPQSQPKKKEKRAYR for generating a HvβCENH3-specific antibody). Peptides synthesized and used for the immunization of different animals (guinea pigs for generating anti-HvαCENH3, and rabbits for generating anti-HvβCENH3 antibodies) that made simultaneous detection of both CENH3-types possible. Peptide synthesis, immunization of animals and peptide affinity purification of antisera was performed by Pineda (Antikörper-Service, Berlin, Germany).

To verify the specificity of the generated antibodies, Western blot analysis was performed using nuclear proteins isolated from young *H. vulgare* leaves. The predicted molecular mass for the HvαCENH3 protein is approximately 18 kDa and for HvβCENH3 is 16 KDa. Figure 22 shows that anti-HvαCENH3 and anti-HvβCENH3 antibodies recognized specific proteins with correct molecular mass. The notable intensity difference between HvαCENH3- and HvβCENH3-specific bands after Western detection (Fig.22 lane 2 and 3) is most likely caused by the different affinity of antibodies. The anti-grassCENH3 antibody was used as internal control (Fig. 22, lane 1). Notably, this antibody revealed a minor and a major band (between 15 and 25 KDa). Comparable Western bands were identified with HvαCENH3- and HvβCENH3-specific antibodies. Therefore, it is likely that anti-grassCENH3 recognizes both HvαCENH3 and HvβCENH3 proteins.

The observed intensity difference of Western bands could be due to presence of different amounts of HvαCENH3 and HvβCENH3 in the protein sample.

Alternatively, regardless of the homology between the sequence of the peptide used to raise the antibody and the barley CENH3 amino acid sequence (HvαCENH3 *versus* grass CENH3 peptide 17 of 21 amino acid are identical; HvβCENH3 *versus* grass CENH3 peptide11 of 21 amino acid are identical), the grass CENH3 antibody has a higher affinity for HvαCENH3 than for HvβCENH3. However, it is less probable, as applying the grass CENH3 antibody on similar amounts of *in vitro* synthesized HvαCENH3 and HvβCENH3 proteins (Fig. 23A) shows that anti-grass CENH3 recognised both proteins with equal affinity. However, it is unknown whether the structure and posttranslational modifications differ between *in vitro* synthesized and native proteins and therefore influencing the binding affinity of antibodies.

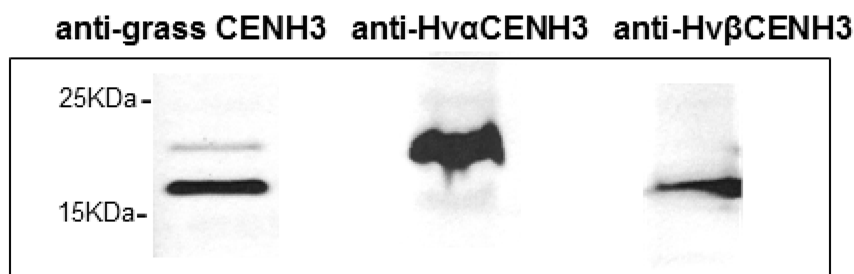


Fig. 22. Western blot assay using grass CENH3-, HvαCENH3- and HvβCENH3-specific antibodies on equal amounts of proteins isolated from leaf nuclei of *H. vulgare*.

In order to test the specificity of each antibody *in vitro* translated proteins were generated. Therefore, the coding sequences of HvαCENH3 and of HvβCENH3 were amplified and translated *in vitro* using the PURExpress cell-free transcription-translation system (New England Biolabs). After PAGE electrophoresis and Western transfer the *in vitro* translated CENH3 proteins were assayed using the generated HvαCENH3 and HvβCENH3 antibodies. In addition, the anti-grass CENH3 antibody was tested on the synthesised proteins (Fig. 23).

Figure 23B shows that anti-HvαCENH3 antibody is αCENH3-specific and is not cross-reacting with the βCENH3-type. Similarly, the anti-HvβCENH3 antibody is

βCENH3-specific (Fig. 23C). The anti-grass CENH3 antibody cross-reacts with both types for CENH3s (Fig. 23A).

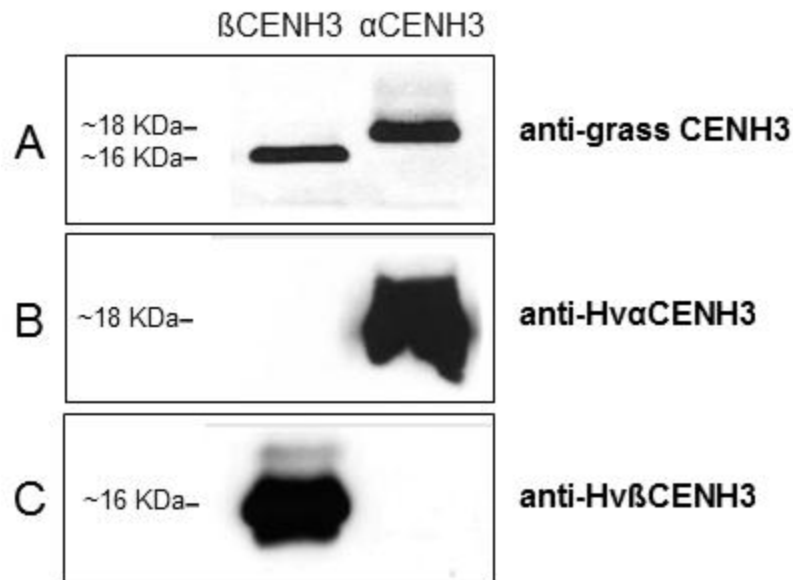


Fig. 23. Western blot assay using (A) anti-grass CENH3, (B) anti-HvαCENH3 and (C) anti-HvβCENH3 antibodies on *in vitro* synthesized αCENH3 and βCENH3 proteins of *H. vulgare*. Result shows that anti-αCENH3 and anti-βCENH3 antibodies are CENH3-type specific and anti-grass CENH3 antibody cross reacts with both CENH3 types.

4.6.1.1. HvαCENH3- and HvβCENH3- antibodies are *H. vulgare*-specific

In order to test anti-HvαCENH3 and anti-HvβCENH3 for species-specificity we performed immunostaining experiments on mitotic root tip cells of *H. vulgare*, *T. aestivum* and *H. bulbosum* (Fig. 24 and 25). The anti-grass CENH3 antibody was used as positive control. Result shows that both, anti-HvαCENH3 and anti-HvβCENH3 are cross-reacting with the CENH3s of barely (Fig. 25), but not with CENH3 proteins of wheat or *H. bulbosum* (Fig. 24) Therefore, HvαCENH3- and HvβCENH3 antibodies are barley specie -specific.

4.6.2. Colocalization of α and ß CENH3 on interphase, mitotic and meiotic chromosomes of *H. vulgare*

To determine the targeting of the α and βCENH3 proteins in barley centromeres immunofluorescence experiments were done on mitotic root tip cells and meiotic

pollen mother cells of *H. vulgare*. We expected to find a “chromosome-type-specific” position for each CENH3 type. As one of the two CENH3 variants of *C. elegance* (CeCENP-A)
was not detected on early meiotic prophase chromosomes (Monen et al., 2005) it was also
likely to find differences between meiotic and mitotic chromosomes of barley regarding CENH3.

Double immunostaning with anti-HvαCENH3 and anti-HvβCENH3 antibodies on somatic nuclei (Fig. 27A) and on chromosomes at different stages of mitosis (Fig. 25) revealed that HvαCENH3 and HvβCENH3 are detectable in the centromeres of all barley chromosomes in all mitotic stages. Signals of both CENH3 variants always overlap with the position of the centromeres.

Similarly, both types of CENH3 were detectable at different stages of first and second meiotic division (Fig. 26). Based on this observation we conclude that HvαCENH3 and HvβCENH3 incorporation is not “chromosome-type-specific” or “cell division type-specific”. Hence, both CENH3 variants of barely are equally involved in the formation of functional mitotic or meiotic centromeres.

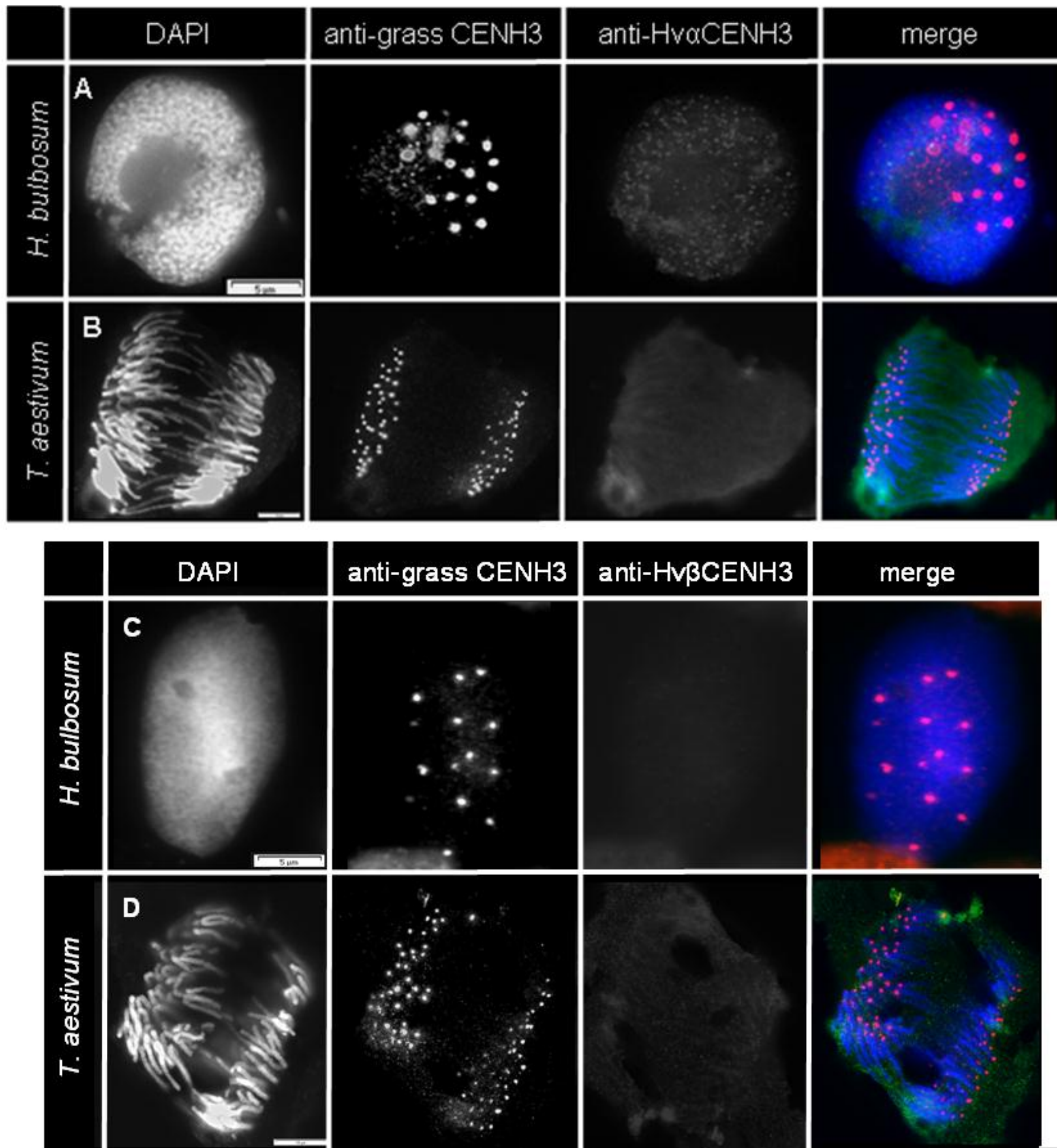


Fig. 24. Double immunostaining of interphase nuclei of *H. bulbosum* and anaphase chromosomes of *T. aestivum* with (A) anti-grass CENH3 (in red) as control and anti-HvαCENH3 (in green) specific antibodies and (B) anti-grass CENH3 (in red) as control and anti-HvβCENH3 (in green). Pictures show that anti-HvαCENH3 and -HvβCENH3 are not cross-reacting with CENH3s of *H. bulbosum* or wheat. Chromosomes are counter-stained with DAPI (in blue). Scale bars present 5 µm.

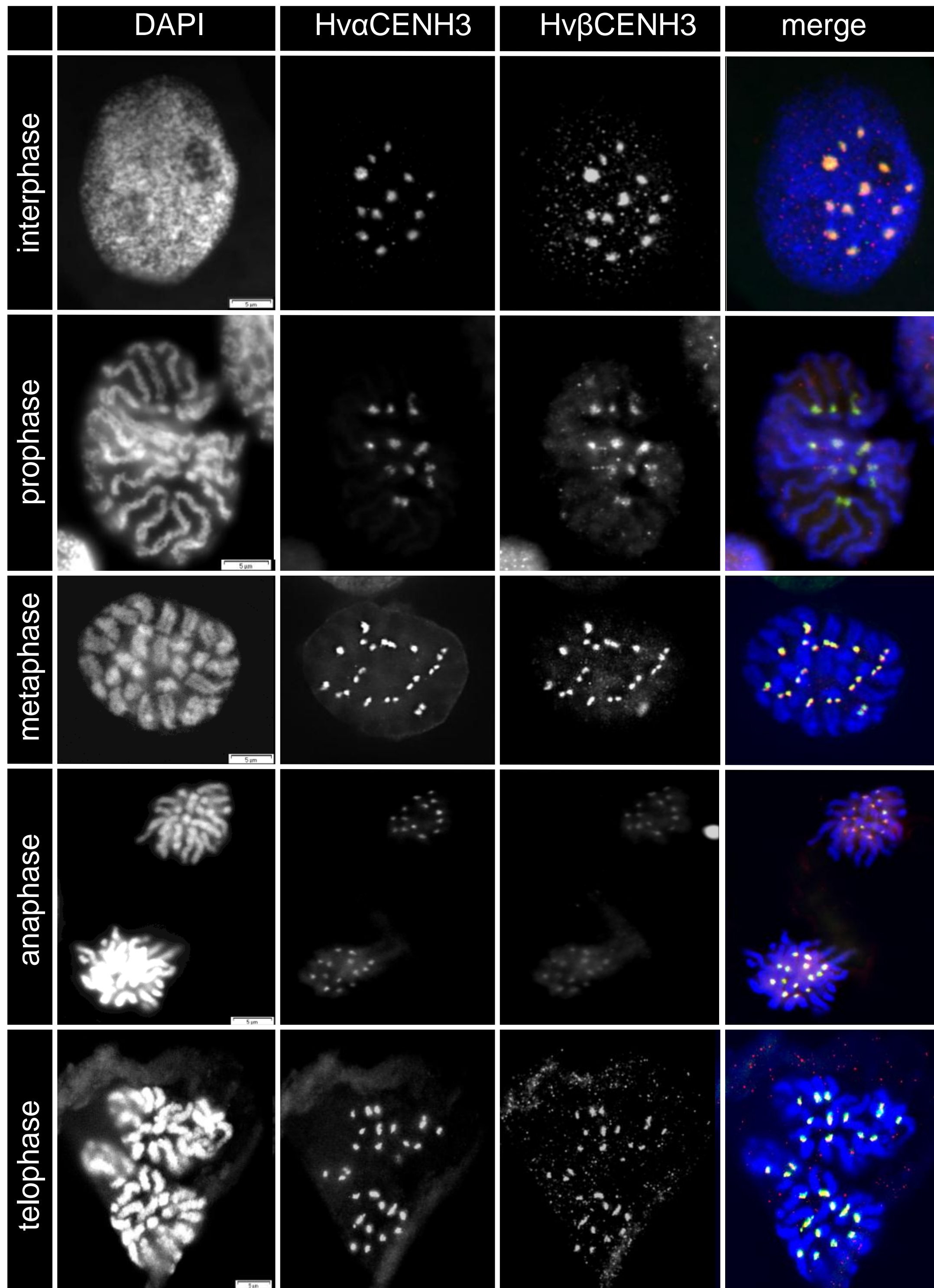
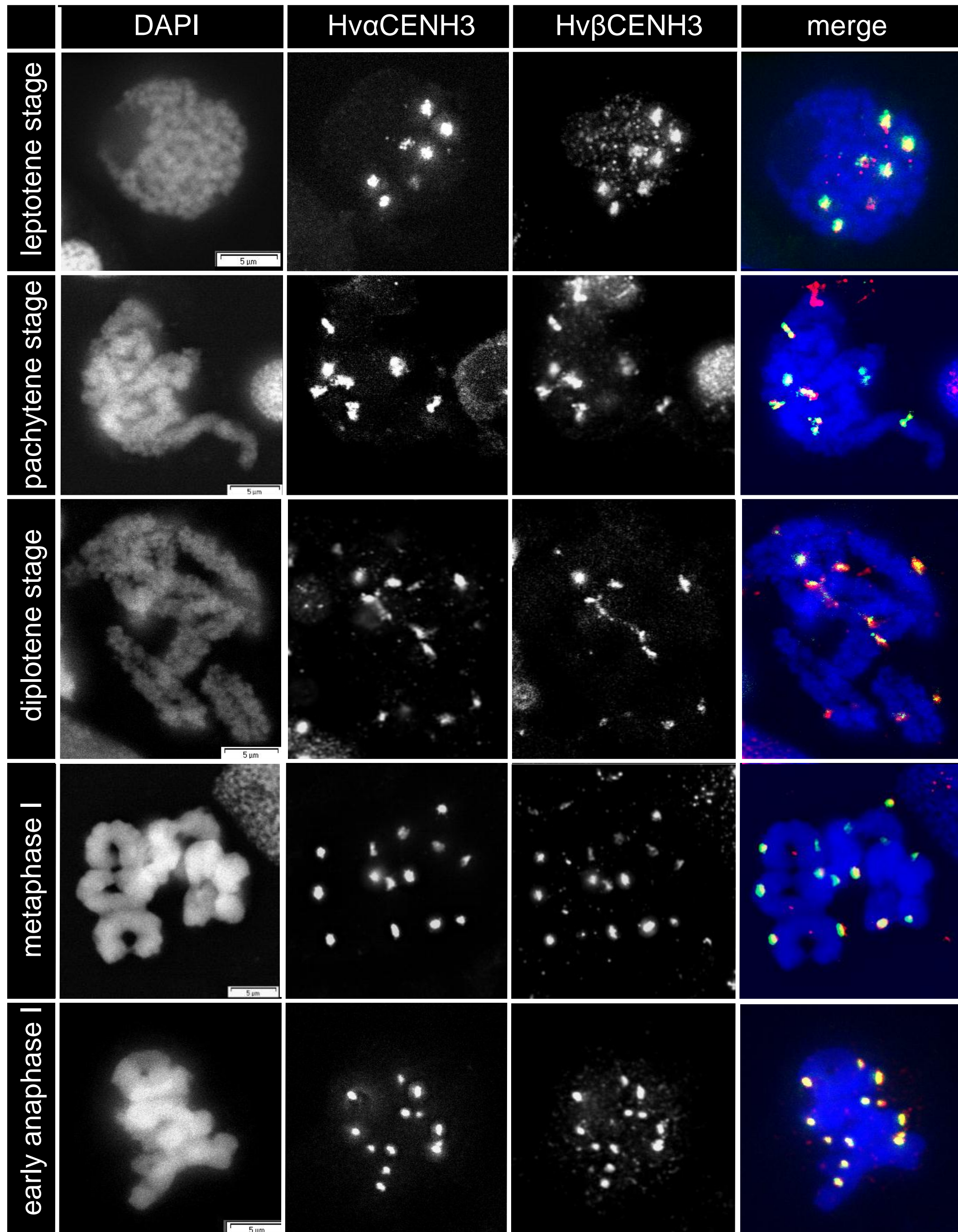


Fig. 25. Double immunostaining of interphase and mitotic cells of *H. vulgare* with HvαCENH3- (in green) and HvβCENH3-specific (in red) antibodies. An overlap of α and βCENH3-signals was found in interphase nuclei, prophase, metaphase, anaphase and telophase stages. Chromosomes are counter-stained with DAPI (in blue). Scale bars present 5 µm.


DAPI
HvαCENH3
HvβCENH3
merge
leptotene stage
pachytene stage
diplotene stage
metaphase I
early anaphase I

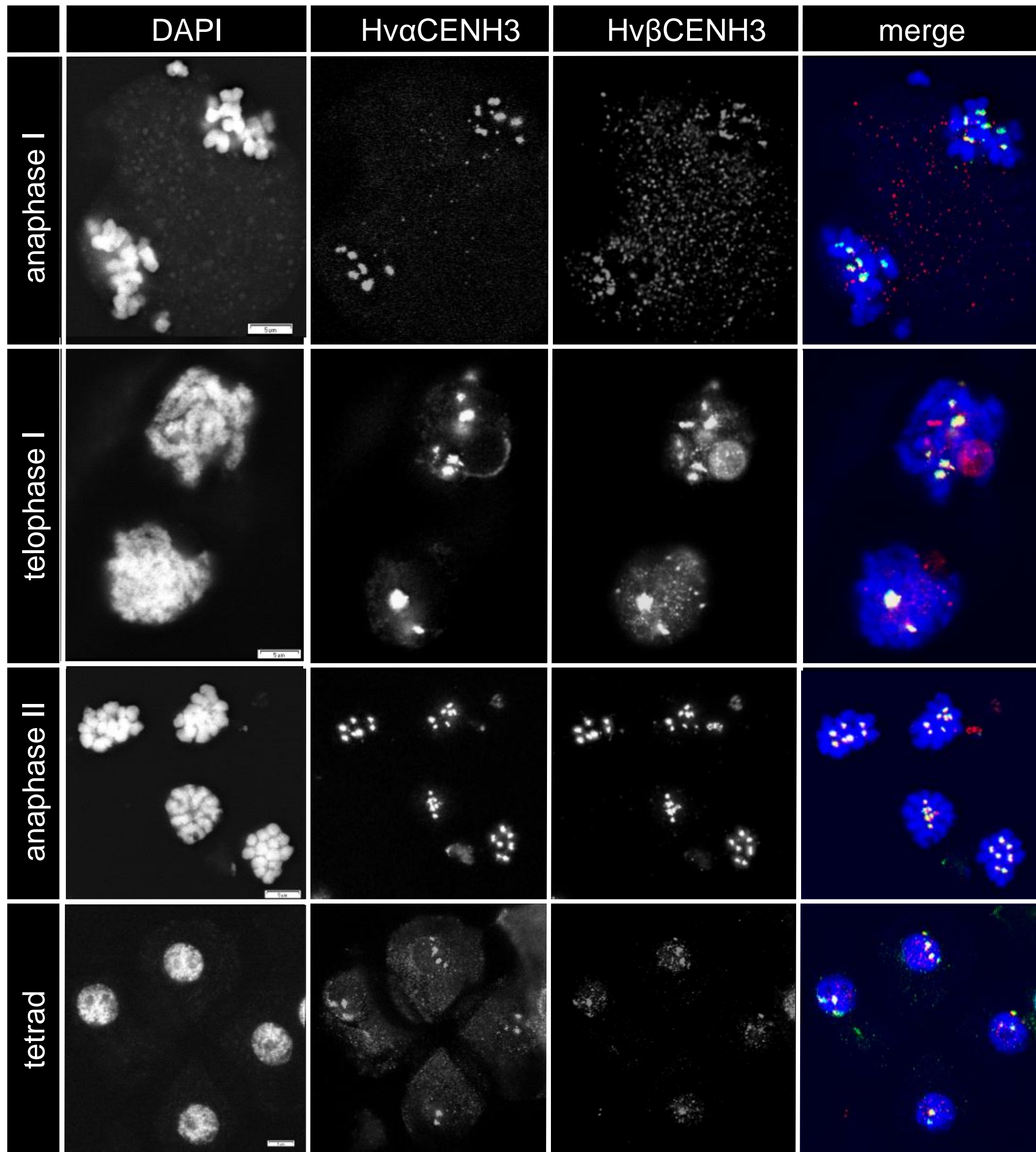


Fig. 26. Double immunostaining of meiotic chromosomes of *H. vulgare* with HvαCENH3- (in green) and HvβCENH3-specific (in red) antibodies. Pictures show CENH3 signals in first prophase stages (leptotene, pachytene, diplotene), metaphase I, early anaphase I, anaphase I, telophase I, anaphase II and tetrad. An overlap of α and βCENH3-signals was found in all meiotic stages. Chromosomes are counter-stained with DAPI (in blue). Scale bars present 5 µm.

4.6.3. αCENH3 and βCENH3 containing nucleosomes are closely intermingled in barley centromeres

In order to decipher the higher-order organization of αCENH3 and βCENH3 containing chromatin immunostaining experiments were performed on extended chromatin fibers. Therefore, chromatin fibers prepared from isolated nuclei of barley leaves were immunolabelled with both types of barely CENH3 antibodies. An overlap of αCENH3- and βCENH3-specific signals was found for unextended interphase chromatin (Fig. 27A), but also for up to12-fold extended chromatin (Fig. 27 B-D). This observation indicates that both variants of CENH3 are intermingled throughout the centromeres of barley. However, it remains unknown whether αCENH3 and βCENH3 are colocalizing to the same or different centromeric nucleosomes.

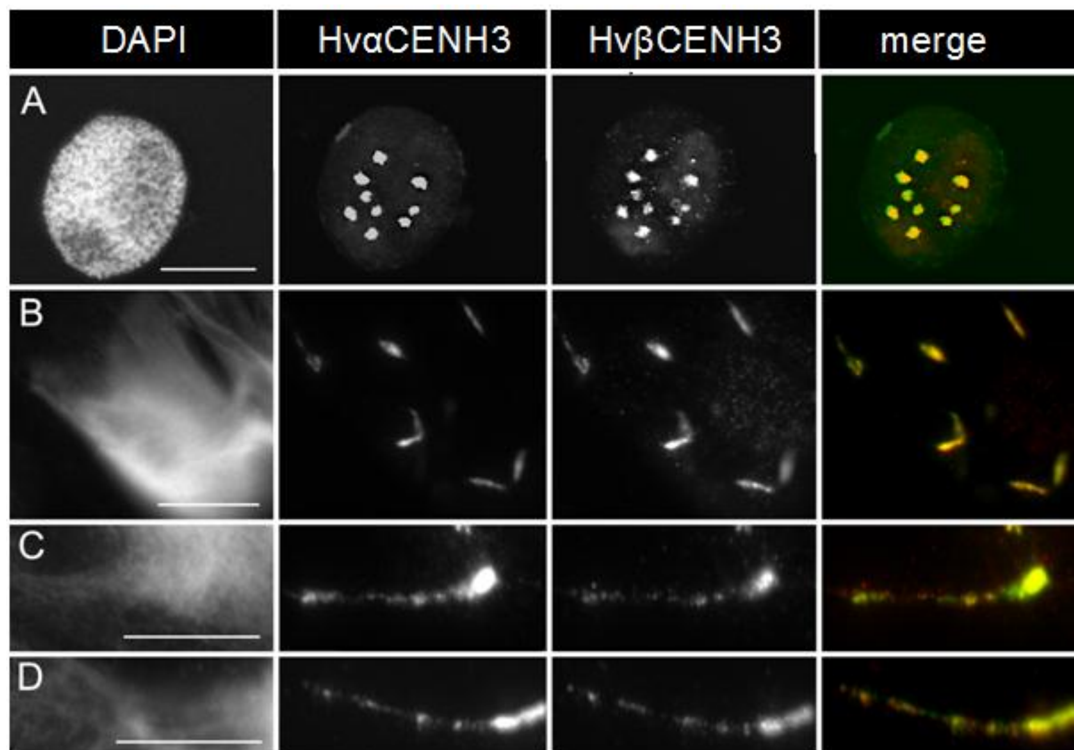


Fig. 27. Immunostaining of nuclei (A) before and (B-D) after chromatin fiber extension with antibodies specific for HvαCENH3 (in green) and HvβCENH3 (in red). (B) Shows a nucleus with CENH3-signal clusters after chromatin fiber extension. (C and D) Depict immunofluorescence signals of centromeres after chromatin fiber extension. Irrespectively of the degree of chromatin fiber extension an overlap of α and βCENH3-specific signals is detectable. Bars indicate 10 µm.

4.7. Estimation of nuclear genome size of *H. vulgare* and *H. bulbosum* genotypes

To test whether the proposed asynchrony of CENH3 loading in unstable hybrids is caused by the different time duration to replicate the genomes of *H. vulgare* and *H. bulbosum,* we investigated their genome size. This assumption was based on previously published data on the correlationship between genome size and DNA replication time duration (Bennett, 1987). Intraspecifc genome size variations were reported for *Hordeum* species (Jakob et al., 2004).

As the parent combination *H. vulgare* x *H. bulbosum* (genotype 2920/4) caused chromosome elimination in hybrids and the parent combination *H. vulgare* x *H. bulbosum* (genotype 3811/3) caused chromosome retention in hybrids, we expected to find a larger genome size difference between *H. bulbosum* (2920/4) and *H. vulgare* than between *H. bulbosum* (3811/3) and *H. vulgare*. Therefore, nuclei were isolated from leaves and measured by flow cytometery. For *H. vulgare* we determined a 2C DNA value of 10.34 pg. The genome size of both *H. bulbosum* genotypes is almost identical, 2C DNA value for 2920/4 is 8.72 pg and for 3811/3 we determined a genome size of 8.88 pg. Therefore, it is less likely that a genome size difference between both *H. bulbosum* genotypes is causing the observed instability of hybrids. Also, the expected delay of DNA replication in *H. bulbosum* does not fit with its smaller genome size.

4.8. *Hordeum* species and their stable hybrids have similar loading behavior for CENH3s

We speculated that elimination of *H. bulbosum* chromosomes in unstable hybrid is due to disordered loading of CENH3s into centromeres of *H. bulbosum*, Therefore, we supposed that the loading behavior of CENH3 in barley and *H. bulbosum* might differ. In *A. thaliana* and barley, CENH3 loading occurs before mitosis and separation of sister kinetochores (Lermontova et al., 2007). It was reported that loading of total CENH3 occurs in barley during late G2 phase

(Lermontova et al., 2007), but the loading behavior of each HvCENH3 variant and the for *H. bulbosum* was not known.

To test this hypothesis, nuclei at different stages of interphase (G1, S and G2) were sorted from isolated from root tip meristems of barley, *H. bulbosum* and stable hybrid plants, using flow cytometry. Relative amounts of CENH3 were determined by measuring the fluorescent signal intensity and size of CENH3 after immunostaining (Fig. 28A). We investigated the loading time of HvαCENH3 and HvβCENH3 proteins in barley and stable hybrid plants using HvαCENH3 and HvβCENH3-specific antibodies. The loading of total CENH3 in *H. bulbosum* (as anti-HbαCENH3 and -HbβCENH3 antibodies are not available) was determined using an anti-grass CENH3 antibody. Measurement of speckle fluorescence intensity and size from single optical sections was performed using TINA 2.0 software (Fig. 28B).

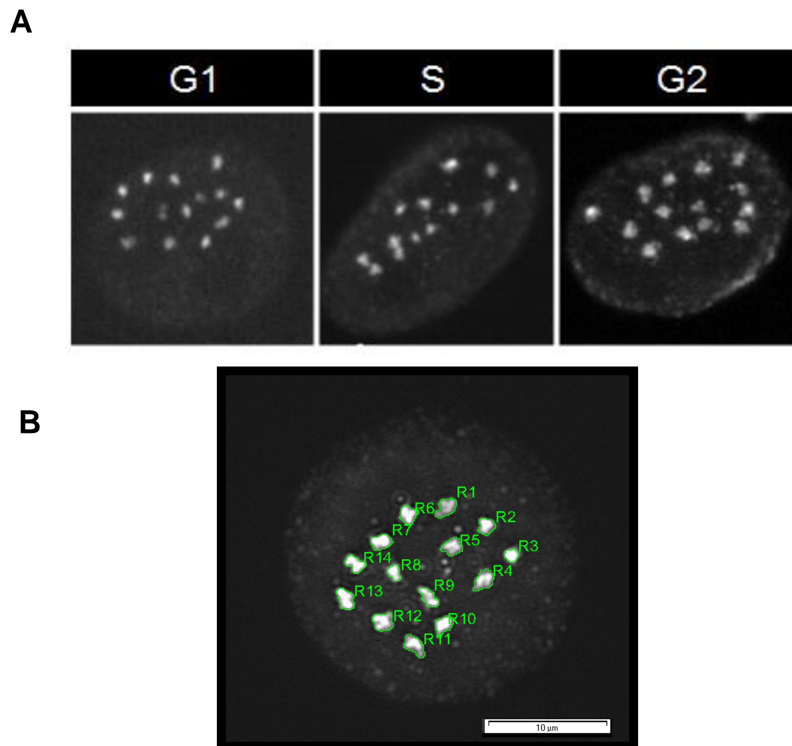


Fig. 28. Analysis of CENH3 loading behavior. (A) Sorted G1, S and G2 nuclei of barley after immunostaining with anti-HvαCENH3 antibody. Note the different signal intensity and size of CENH3-signals at different stages of interphase (G1 versus G2). (B) CENH3 signal size and intensity was determined using TINA software.

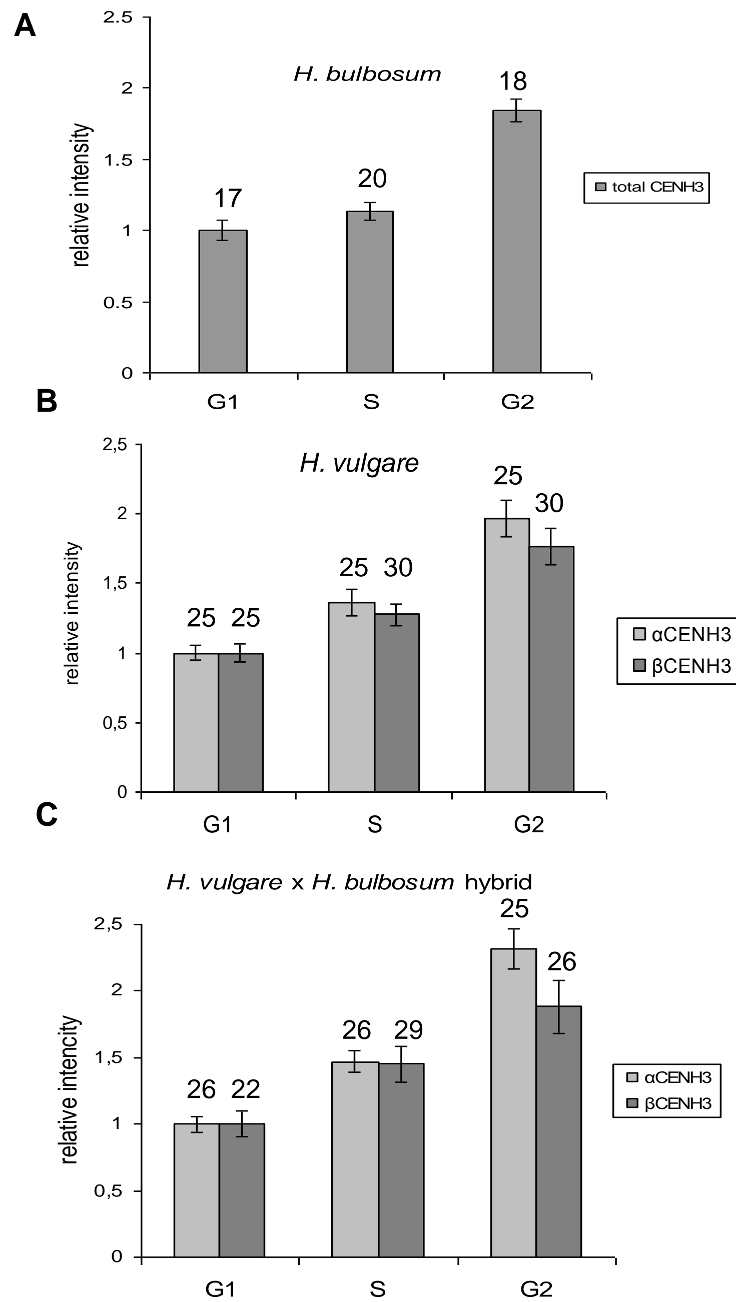


Fig. 29. Analysis of CENH3 loading behavior of (A) *H. bulbosum*, (B) *H. vulgare* and (C) of a stable *H. vulgare* x *H. bulbosum* hybrid. Histograms showing the relative amounts of CENH3 as measured by fluorescence intensity after indirect immunostaining with CENH3s-specific antibodies (± SE). Number of analyzed nuclei for each sample is indicated.

The florescence intensities of CENH3-signal clusters of at least 17 nuclei per genotype, antibody and nuclei-type were measured. Relative CENH3 signal intensities (that includes signal intensity and signal size) were compared between G1, S and G2 nuclei (Fig. 29). An almost doubling of relative intensity of HvαCENH3- and HvβCENH3-signals was observed for G2 nuclei isolated from *H. vulgare* (Fig. 29B) or *H. vulgare* X *H. bulbosum* hybrid plants (Fig. 29C). A comparable increase of relative signal intensity was also observed for grass CENH3-specific signals of *H. bulbosum* (Fig. 29A). These observations suggest that the loading behavior of CENH3 in *H. vulgare, H. bulbosum* and in *H. vulgare* X *H. bulbosum* hybrids is comparable and occurs during G2 mainly. A slight increase of signal intensity at S-phase may indicate that the loading process of CENH3s is starting earlier than G2 phase.

About 10% of *H. vulgare* X *H. bulbosum* G2 hybrid nuclei (Fig. 30) revealed seven big and seven small total CENH3 signals (detected by anti-grass CENH3 antibody). This difference may indicates that CENH3 has a delay in loading in the centromeres of the *H. bulbosum* or it degenerates faster just before reloading in the late G2 phase.

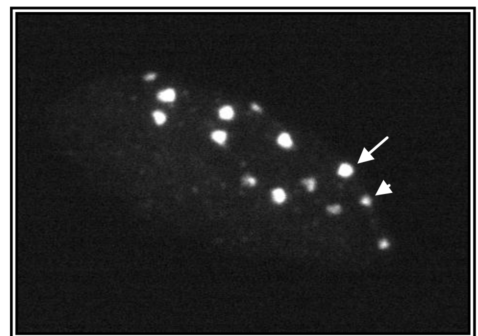


Fig. 30. Two classes of CENH3 signals (after immunostaining with anti-grass CENH3) were observed in *H. vulgare* x *H. bulbosum* hybrid root tip. Arrow and arrow head show big and small signal respectively.

However, it seems that CENH3 targeting in both parental and hybrid species occurs similarly in G2 phase and difference in the loading time of the parental CENH3s is not influencing hybrid stability.

4.9. Incorporation of barley CENH3 proteins in stable addition lines and hybrids

To investigate the incorporation behavior of *H. vulgare* CENH3s into the centromeres of alien chromosomes in stable addition lines or hybrids we studied three different situations.

1- When an alien chromosome without corresponding CENH3 gene substitutes a chromosome of barley. Therefore, we used a *H. vulgare*-*H. bulbosum* 7H substitution line.

2- In the combination of two different genomes, therefore, we studied a stable *H. vulgare* x *H. bulbosum* hybrid.

3- When barley chromosomes carrying CENH3 genes are added to the genome of a less related species, for this we employed different wheat-barley double disomic addition lines of 1H + 6H.

4.9.1. α and ßCENH3 of barley compensate missing CENH3s of *H. bulbosum*

To investigate whether CENH3s of barley incorporate into centromeres of alien chromosomes of a closely related species, a *H. vulgare*/*H. bulbosum* 7H substitution line was studied. Chromosome 7H of *H. bulbosum* does not carry any CENH3 gene, as the CENH3 genes of *H. bulbosum* are located on chromosome 1H and 6H. Further, we were interested to analyze whether both or only one variant of HvCENH3 incorporates into centromeres of *H. bulbosum.*

To confirm the genotype mitotic metaphase cells of the putative *H. vulgare*/*H. bulbosum* 7H substitution line were *in situ* hybridized with labeled genomic DNA of *H. bulbosum* to detect the substituted chromosome from *H. bulbosum* (in green), with a centromere repeat (AGGGAG)n that labels specifically *H. vulgare* centromeres but not *H. bulbosum* centromeres (in yellow) (Appendix Fig. 3

shows the specificity of the centromeric repeat), and with 5S rDNA for the identification of each barley chromosome type (in red). Figure 31 shows one chromosome pair with *H. bulbosum*-specific signals but not with centromere-specific signals of *H. vulgare.* The other six chromosome pairs were identified as chromosome 1H-6H of barley based on the barely 5S rDNA-specific hybridization pattern (Fukui et al., 1994). Thus we conclude that *H. bulbosum*-positive labeled chromosome pair is chromosome pair 7H of *H. bulbosum.* Hence, the plant material used represents a *H. vulgare* /*H. bulbosum* 7H substitution line.

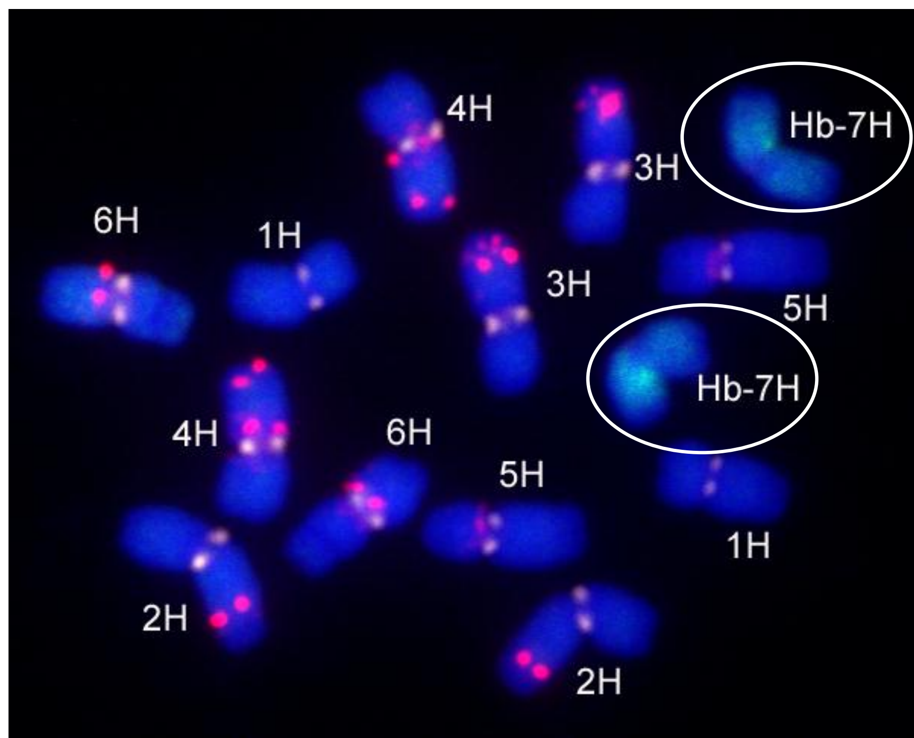


Fig. 31. *H. vulgare* -*H. bulbosum* 7H substitution line after FISH using genomic *H. bulbosum* DNA (in green), (AGGGAG)n a barley specific centromere repeats (in yellow) and 5S rDNA (in red) as probes. Circles indicate chromosome 7H of *H. bulbosum.* Scale bar presents 10 µm.

Next double immunostaining on root tip nuclei of the characterized substitution line with grass CENH3- (as positive control), HvαCENH3- and HvβCENH3-specific antibodies were performed (Fig. 32). For each CENH3 antibody used up to 14 signals per nucleus were detected. Hence, both α and ßCENH3 of barley

are able to incorporate into the centromeres of *H. bulbosum* and can functionally compensate the missing CENH3s of *H. bulbosum*.

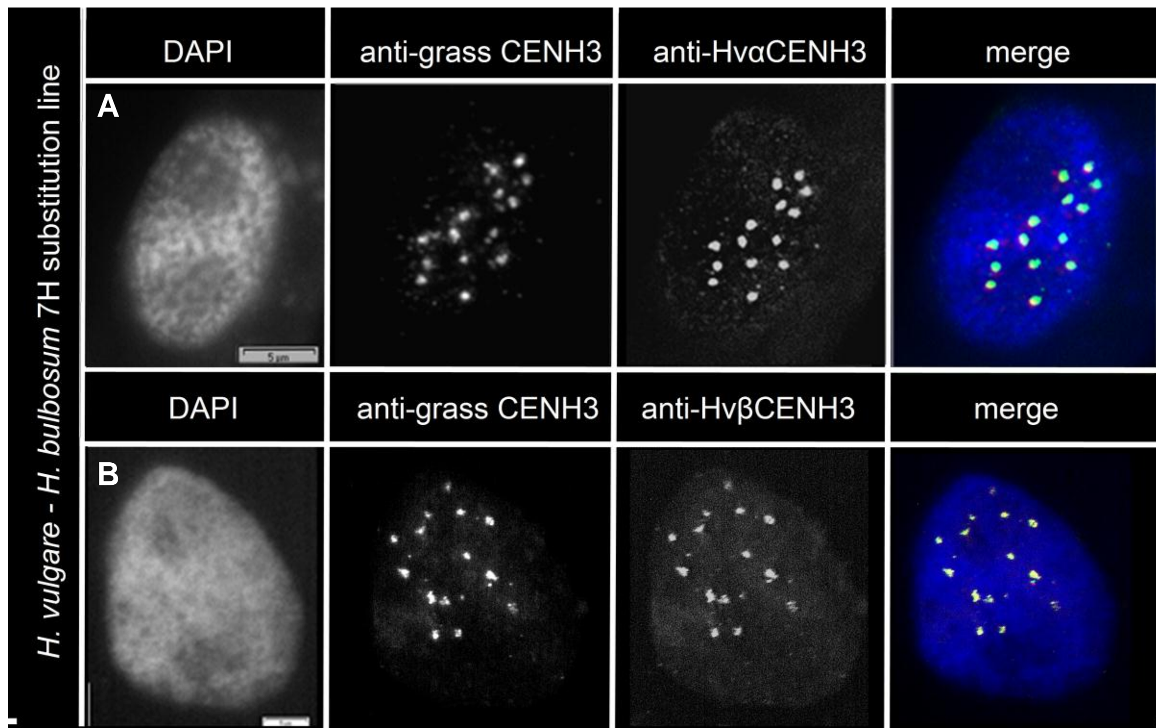


Fig. 32. Double immunostaining of nuclei isolated from a *H. vulgare*/*H. bulbosum* 7H substitution with anti-grass CENH3, anti-HvαCENH3 and anti-HvβCENH3. (A) Combination anti-grass CENH3 (in red) and anti-HvαCENH3 (in green); (B) combination anti-grass CENH3 (in red) and anti-HvβCENH3 (in green). For each CENH3 antibody used up to 14 signals per nucleus are countable. Scale bars present 5 µm.

4.9.2. Centromeres of wheat incorporate HvαCENH3 but not βCENH3 of *H. vulgare*

To determine whether HvCENH3 protein incorporation occurs when barley chromosomes carrying HvCENH3 genes are added to the genome of a less related species we employed different wheat-barley double disomic addition lines of 1H + 6H.

To confirm the genotype and expression of HvβCENH3 genes, PCR experiments were performed with Hvα and βCENH3-specific primers (primers 6 and 16 for HvαCENH3 and primers 4 and 8 for HvβCENH3) on gDNA and cDNA of the putative wheat-barley addition lines of 1H + 6H. GAPDH amplification was used as positive control. Result shows that HvαCENH3 (locates on 1H of barley) and HvβCENH3 (locates on 6H of barley) genes present in the candidate plant and transcribe as well (Fig. 33). Thus, the wheat-barley addition lines analyzed are carrier 1H and 6H chromosomes of barley and both genes are active in the wheat background as well.

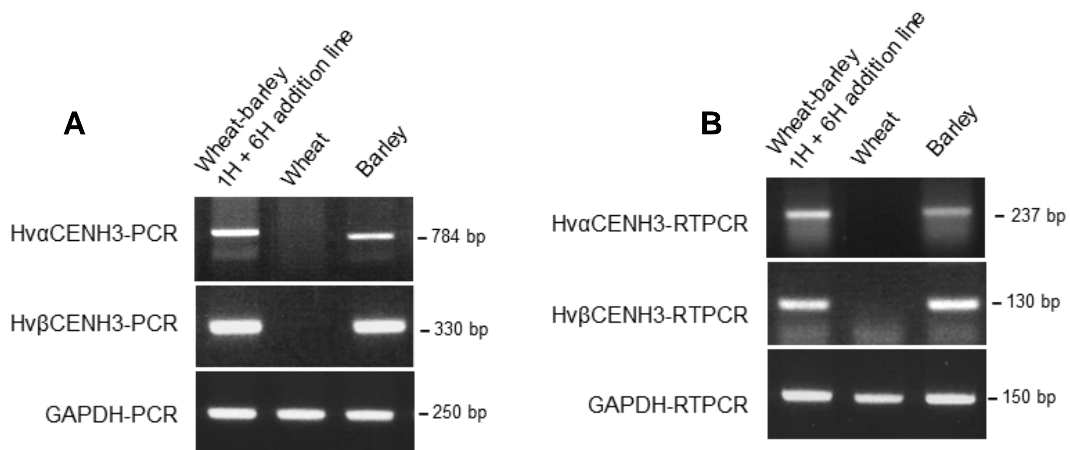


Fig. 33. Amplification of HvαCENH3 and HvβCENH3 in the wheat-barley double disomic addition line of 1H + 6H with barley CENH3 specific primer pairs. GAPDH primers were used as positive control. (A) PCR using genomic DNA confirmed the presence of 1H and 6H chromosomes and (B) RT-PCR demonstrated transcription of HvαCENH3 and HvβCENH3 in the wheat background.

Next double immunostaining on root and leaf nuclei of the characterized wheat-barley addition lines with grass CENH3 (as positive control), HvαCENH3 and HvβCENH3-specific antibodies were performed (Fig.34). For HvαCENH3 up to 46 signals per nuclei were detected in nuclei isolated from roots or leaves. However, no HvβCENH3 signals were detectable in either of the nuclei although the HvβCENH3 gene is transcribed. Hence, centromeres of wheat incorporate HvαCENH3 but not βCENH3 of *H. vulgare* despite transcription.

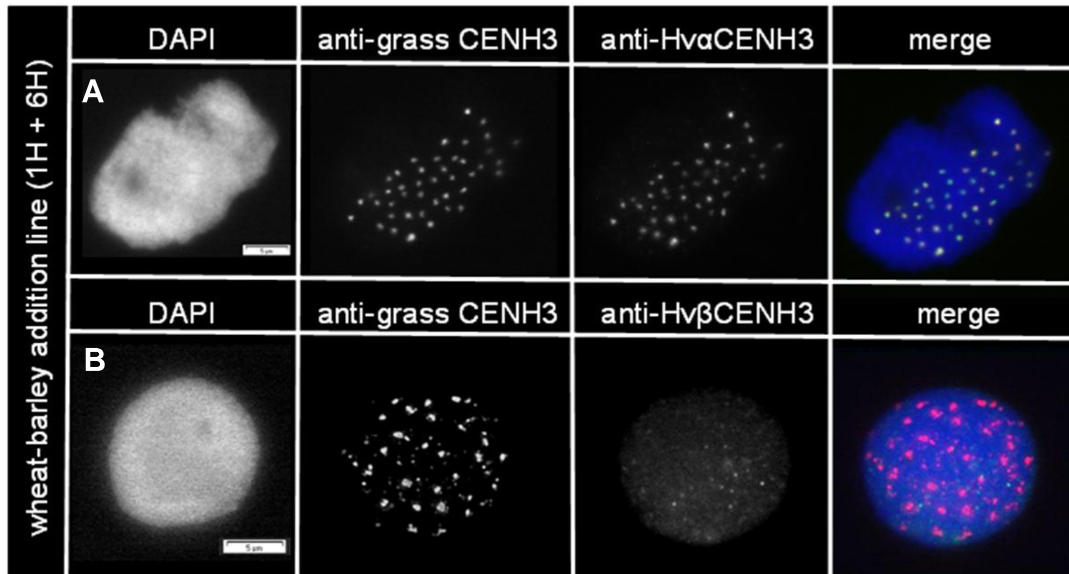


Fig. 34. Double immunostaining of nuclei isolated from leaf and root of wheat-barley double disomic addition lines of 1H + 6H. (A) Shows combination of anti-grass CENH3 (in red) and anti-HvαCENH3 (in green); (B) show combination anti-grass CENH3 (in red) and anti-HvαCENH3 (in green). HvαCENH3 overlaps with the grass CENH3 signals. Indicating that the HvαCENH3 incorporates into the centromeres of wheat as well as of barley. No HvβCENH3-signals were detectable at all. Result was similar in root and leaf tissues. Chromosomes are counter-stained with DAPI (in blue). Scale bars present 5 µm.

5. Discussion

5.1. Duplication of the CENH3 gene in *Hordeum* species

This is the first report about two functional centromeric histone H3 variants in diploid grasses. Other known diploid plants with more than one CENH3 gene are *Arabidopsis lyrata* and *A. halleri* (Kawabe et al., 2006) with two identified CENH3 genes. Both genes locate on the same chromosome, and the second CENH3 copy was generated from a recent duplication probably following duplication of newly arisen centromeric sequences. The only known mammal with more than one CENP-A gene (the mammalian CENH3 homolog) is cow (*Bos primigenius Taurus*). For cow a CENH3 gene family with 11 members was reported. As CENH3 is a single copy gene in all other mammals examines and CENH3 members are highly similar in cow, it is speculated that the gene copy expansion is a very recent event in this species (Li and Huang, 2008). All CENH3 like gene members in cow are expressed, but it is predicted that most of them are non-functional as many terminator mutants were observed in their open reading frame (Li and Huang, 2008).

The other known case of CENH3 gene duplication in a diploid species was reported for the nematode *Caenorhabditis elegans,* which has two CENH3 loci, but the second copy is expressed only very weakly (Monen et al., 2005). On the other hand, the closely related species *C. briggsae* encodes only one CENH3 variant, indicating that the second *C. elegans* gene is derived from a recent duplication event.

As expected, allopolyploid organisms encode multiple CENH3 types. For example, the allotetraploid species *Nicotiana tabaccum* (Nagaki et al., 2009) and *Arabidopsis suecica* (Talbert et al., 2002) encode two CENH3s that are highly similar to the CENH3s of their ancestral diploid species. In two allotetraploid *Oryza* species two CENH3 genes were also identified, which are both transcribed (Hirsch et al., 2009). However, in *Zea mays*, which is a fairly recent allotetraploid species (due to an event estimated to be 11 million years ago (MYA) formed

(Gaut and Doebley, 1997)), only one centromeric histone H3 gene copy was identified (Zhong et al., 2002). However, for none of the species with multiple CENH3s, except for *H. vulgare,* CENH3 variant-specific antibodies were generated.

5.1.1. *Hordeum* αCENH3 is the ancestral variant and βCENH3 was generated by a duplication of the α type

We identified two CENH3 types in all *Hordeum* species analysed, namely *H. vulgare*, *H. bulbosum* and *H. marinum*. In addition, we have evidence for the existence of two CENH3 types in *Secale cereale* and of at least six CENH3 types in hexaploid wheat as well (Houben, personal communication). Rice encodes only one CENH3 gene. Also, TBLASTX search analysis of the complete genomic sequence of *Brachypodium distachyon* (http://www.modelcrop.org/index.html), revealed only one CENH3 gene (Bradi2g21810.1). Thus the CENH3 gene duplication is an old event and probably occurs after divergence of *Brachypodium* from Triticaea tribe (which includes the majority of important temperate cereals and forage grasses) approximately 12 MYA (Fig. 35).

Evidence exists that a large scale, perhaps genome-wide duplication occurred 20 million years before the divergence of *Oryza*, *Sorghum*, and *Hordeum* from common ancestors that existed 41–47 MYA (Paterson et al., 2004; Thiel et al., 2009). It is possible that the second variant of CENH3 is a remain of an ancestral whole genome duplication event, while the duplicated CENH3 gene has been removed from the rice and *B. distachyon* genomes, likely via selection. But, this hypothesis is less likely as 1H and 6H chromosomes of barley do not share any duplicated segment (Thiel et al., 2009).

Our analysis indicates that αCENH3 is the ancestral variant and that βCENH3 was generated by the duplication of the α type. This is because αCENH3 of *H. vulgare* and *H. bulbosum* mapped to chromosome 1H, and 1H of *H. vulgare* has synteny with the chromosome 5 of rice (Thiel et al., 2009) and 2 of *Brachypodium*

(Initiative, 2010). On other hand CENH3 of rice locates on chromosome 5 (Nagaki et al., 2004) and CENH3 of *Brachypodium* located on chromosome 2. In addition *Brachypodium* and rice CENH3 has greater sequence similarity to αCENH3 of *H. vulgare* and *H. bulbosum* than to the βCENH3 type. The phylogenetic tree of CENH3s shows a clustering of *Hordeum* αCENH3s with CENH3 of rice and of other grasses (Fig. 10).

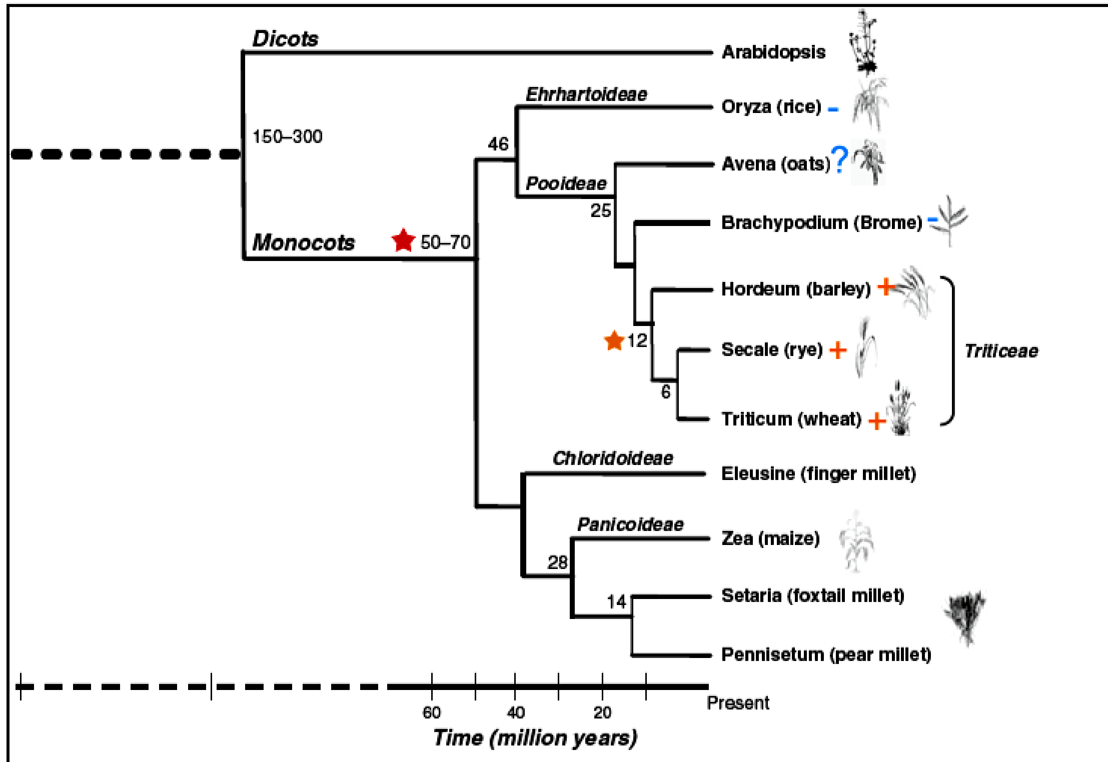


Fig. 35. Phylogenetic relationship between grasses and Triticeae species. Divergence time from common ancestor are indicated on the branches of the phylogenetic tree (in million years) (Dvorak, 2009). (+) and (-) indicate species with and without duplicated CENH3 genes, respectively. Stars indicate duplication events of CENH3.

5.1.2. Divergence of α and βCENH3 types

Although often duplicated genes are not functional and under a high rate of mutation during the evolution, a duplicate gene could gain a new function, and thus contribute to the diversity of the genome. Duplicated genes might divide up the ancestral function, so that, rather than acquire new functions; they simply became subfunctionalized (Lynch and Force, 2000). However, in the case of *H.*

vulgare and *H. bulbosum* interspecific similarity is much larger than intraspecific similarity between α and βCENH3s. Therefore it is possible that the duplicated gene has a conserved function which was under selection pressure. We confirmed this idea by the application of specific antibodies against αCENH3 and βCENH3 of *H. vulgare* that both showed centromere-specific localization patterns.

Our study shows that both CENH3 types of barely are present in all chromosomes at different stages of meiosis and mitosis as well as in nuclei of different tissue types. With the resolution of the extended chromatin fibers we could not find any differences between α and βCENH3 regarding the interaction with different subcentromeric regions. But, still it remains to study whether both CENH3s load into the same or different centromeric nucleosomes. Also, as the centromere targeting domain (CATD) region, which is critical for centromeric localization of CENH3 (Shelby et al., 1997; Vermaak et al., 2002), differs between of α and βCENH3 of *Hordeum* species (Fig. 9), each CENH3 variant might interact with different centromeric DNA sequences (Shelby et al., 1997; Vermaak et al., 2002b) as well as different chaperones (Foltz et al., 2009; Shuaib et al., 2010) or other 'loading/maintenance factors', which are essential for correct centromeric deposition of CENH3 (Carroll et al., 2009; Lagana et al., 2010).

There are contradictory reports about centromeric nucleosome structure. An atypical structure was proposed for the centromeric nucleosomes of fruit fly (Dalal et al., 2007) and human (Dimitriadis et al., 2010). Here, an asymmetric heterotypic tetramer, a “hemisome” consisting of only one molecule of each CENH3, H4, H2A and H2B, rather than the canonical octamer with two copies of each were suggested. CENH3 nucleosomes of fruit fly, which were measured by atomic force microscopy, yielded only half of dimension of canonical nucleosomes (Dimitriadis et al., 2010). On the other hand, other studies confirmed an interaction of CENH3 with a second CENH3 and the formation of homodimers *in vivo* (Camahort et al., 2009) and *in vitro* (Erhardt et al., 2008).

These data suggest that in budding yeast, fruit fly and human, CENH3 also exists as an octamere nucleosome containing two copies each of CENH3, H2A, H2B and H4 (Sekulic et al., 2010).

In human, CENH3 loading occurs in telo/G1 phase, centromeric DNA is replicated during S phase, in which parental CENH3 nucleosomes are distributed to daughter strands. Therefore, chromatin at the centromeres contains one half of the complement of CENH3 nucleosomes after the completion of S phase and during subsequent G2 and m phases. One might speculate that ‘unusual’ CENH3 containing nucleosomes represent centromeric chromatin in an intermediate state in non plant organisms that contains one half of the amount of CENH3, before it is fully replenished with new CENH3 molecules later in the cell cycle (Probst et al., 2009). However, in plants the situation is different, as new CENH3 targeting occurs late G2, thus it fills the CENH3 gapes that remained after DNA replication before entering mitosis.

If we consider centromeric nucleosomes in plants as canonical octamers, it is possible that in *Hordeum* species both CENH3 variants load into the same nucleosomes (Fig. 36B). In human, the C-terminal end stitch of the α2-helix forms a CENH3-CENH3 interface to form an octameric nucleosome. Notably, this interaction region has an almost conserved sequence in all *H. vulgare* and *H. bulbosum* CENH3 variants (FIG. 41). Therefore, an interaction of different CENH3 types in one octameric nucleosome is likely.

Alternatively, different CENH3 variants of *Hordeum* could be deposited to distinct nucleosomes probably interacting with different DNA sequences (Fig. 36A). CENH3 of fruit fly is thought to be coevolving in response to changes in the rapidly evolving centromeric DNA sequences during the evolution of species (Sainz et al., 2003). Similarly, CENH3 change may occurs as a response to the centromeric sequence changes in one species. However, an overlap of αCENH3- and βCENH3-specific signals was found for centromeric chromatin (Fig. 25), also

for up to 12-fold artificially extended centromeres (Fig. 27). This observation indicates that both variants of CENH3 are intermingled throughout the centromeres of barley.

Nevertheless, it remains unknown whether α and βCENH3 are deposited to the same or different centromeric nucleosomes and whether they are interacting with similar or different DNA sequences. Performing chromatin immunopercipitation-sequencing (ChIP-seq) experiments will help to understand whether each CENH3 variant interacts with the same or different centromeric DNA sequences. To test whether α- and βCENH3 are directly interacting and therefore forming octameric nucleosomes tandem-immunopercipitation experiments on isolated mono nucleosomes of barely with HvαCENH3- and HvβCENH3-specific antibodies could be performed in future.

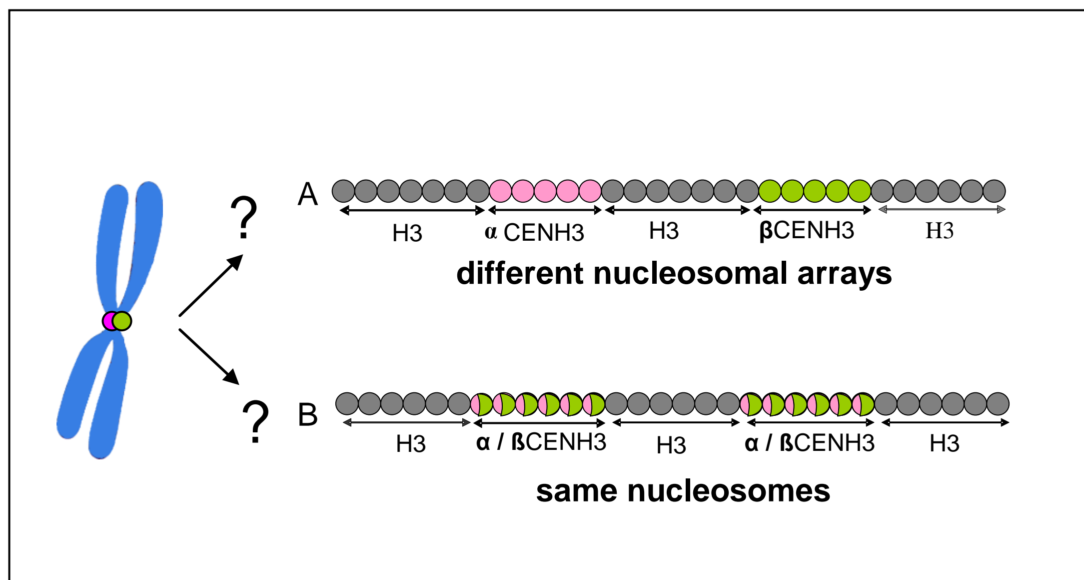


Fig. 36. Model for the possible structure of centromeric nucleosomes in barley. (A) αCENH3 and βCENH3 forming homo-nucleosomes and targeting different centromeric sequences or (B) αCENH3 and βCENH3 form hetero-nucleosomes and targeting the same centromeric DNA αCENH3 is shown in red, βCENH3 in green and histone H3 in gray.

5.2. Incorporation of α and βCENH3 into centromeres of *H. vulgare* and *H. bulbosum* occurs mainly in G2 phase

Canonical histone H3 has been suggested to be deposited into duplicated DNAs during S phase, in a semi-conservative manner (Natsume et al., 2007; Tagami et al., 2004). Experiments using fluorescence recovery after photo bleaching demonstrated that CENH3 of the budding yeast *Saccharomyces cerevisiae* is recruited to centromeres coincident with DNA synthesis (Pearson et al., 2004). Presumably reflecting disassembly and reassembly of centromeric nucleosomes at the replication fork. In contrast, studies performed in human cells (Jansen et al., 2007; Shelby et al., 2000) and in *Drosophila melanogaster* (Ahmad and Henikoff, 2001; Schuh et al., 2007; Sullivan and Karpen, 2001) indicated that CENH3 is incorporated in a replication-independent manner. In fission yeast a biphasic CENH3 incorporation system was reported (Takayama et al., 2008). In this case the incorporation occurs in S and G2 phases. The authors claimed that the G2 deposition of CENH3 acts as a salvage pathway.

In plants CENH3 incorporate to the centromere before mitotic sister centromere separation occurs. In higher plants with monocentric chromosomes like *A. thaliana,* it takes place during late G2 (Lermontova et al., 2007) and in holocentric plants it happens from prophase to metaphase (Nagaki et al., 2005a). Here we report that both CENH3s in *H. vulgare* and *H. bulbosum* recruit to the centromeres mainly during G2 phase as previously reported by (Lermontova et al., 2007). However, we also noticed a low increase of CENH3 signals during the S phase. Therefore biphasic dependent loading machinery like in fission yeast is imaginable in barley too.

5.3. The role of CENH3 in the process of uniparental chromosome elimination in wide hybrids

In unstable *H. vulgare* x *H. bulbosum* hybrid embryo cells, *H. bulbosum* chromosomes show missegregation, defects in attachment of kinetochores to the

microtubules, less chromosome condensation and micronuclei formation. After immunostaining the centromeres of *H. vulgare* are CENH3-postive, while centromeres of *H. bulbosum* revealed no or a reduced amount of CENH3 protein. As CENH3 is a mark for centromere activity it is possible to conclude that uniparental centromere inactivation via loss of CENH3 is the cause of mitosis-dependent chromosome elimination in wide hybrids, as previously postulated by (Finch, 1983; Jin et al., 2004; Mochida et al., 2004).
In unstable hybrid embryos of *H. vulgare* x *H. bulbosum*, CENH3 protein was not detectable in the lagging chromosomes of *H. bulbosum* neither in micronuclei formed by *H. bulbosum* chromatin. In addition, frequently hybrid nuclei with seven weak and seven strong CENH-signals were found. Loss of CENH3 in non-hybrids also results in the failure of centromere formation and kinetochore assembly in mammals (Howman et al., 2000), *C. elegans* (Oegema et al., 2001), fruit fly (Blower et al., 2006), *A. thaliana* (Ravi and Chan, 2010) and in chicken cells (Regnier et al., 2005). In contrast to unstable *H. vulgare* x *H. bulbosum* hybrid embryos, loss of CENH3 in those species is causing mitotic arrest and embryos lethality. It is likely that the centromere activity of *H. vulgare* in unstable *H. vulgare* x *H. bulbosum* hybrid promotes the mitosis and therefore uniparental loss of CENH3 is not embryo lethal.

Complete elimination *of H. bulbosum* chromosomes is finalized 5 to 9 days after pollination (Bennett et al., 1976; Gernand et al., 2006; Subrahmanyam and Kasha, 1973). As CENH3 is a stable protein (Howman et al., 2000) pollen derived CENH3 proteins are likely to provide residual kinetochore function of *H. bulbosum* until failure of chromosome segregation results in chromosome elimination. Fraction of parental H3 variants transmitted to the progeny has been demonstrated for animal embryos (Howman et al., 2000). If pre-existing CENH3 protein is partitioned equally between duplicated sister centromeres (Foltz et al., 2009; Schuh et al., 2007) and no *de novo* incorporation of CENH3 into *H. bulbosum* centromere occurs, its amount will be approximately halved at each cell division (Fig. 37). Reduced CENH3 persist for some cell division as it was

shown that even 10% of the endogenous CENH3 can support efficient kinetochore assembly in human (Liu et al., 2006). On the other hand, a reduced CENH3 amount causes loss of centromeres has been demonstrated for neocentromeres of maize (Topp et al., 2009).

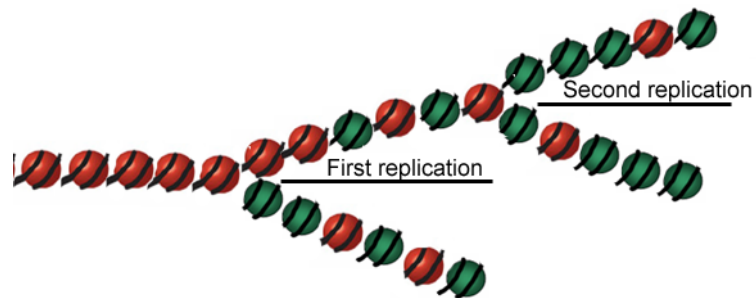


Fig. 37. CENH3 quantitatively redistribute to daughter centromeres contemporaneous with DNA replication. Nucleosomes containing CENH3 are shown in red and histone H3 in green.

Notably, CENH3 null mutant embryos in mice survived for 6.5 days post conception (Howman et al., 2000). Affected embryos showed severe mitotic problems including micronuclei formation and chromatin fragmentation similar to our observations. So this may indicate that despite CENH3 removal at the zygotic stage, still a residual amount of parental CENH3 remains in centromeric nucleosomes which can keep the centromeres active for some cell divisions.
A recent study by Ingouff et al. (2010) on *A. thaliana* demonstrated that, like in animals (Ooi et al., 2006) after fertilization perhaps all parental CENH3 is removed from zygotic nucleus in a DNA replication independent manner and replaced with newly synthesised CENH3 of both parental genomes. However, there is a possibility that residual parental CENH3 is transmitted to the progeny that was not detectable (Ingouff et al., 2010).

A recent study in human stem cells showed that the amount of CENH3 protein required to define an active centromere may be cell type-specific and undifferentiated stem cells (that is in use as a model for embryo development) require less CENH3 protein to faithfully retain the centromeric function relative to

fibroblasts (Ambartsumyan et al., 2010). It is likely that the same is true for plant embryos. Overall it indicates that there is a possibility that the residual CENH3 after fertilization keeps the centromere of *H. bulbosum* active for some cell divisions.

Based on our initial observation we assumed first that the CENH3 genes of *H. bulbosum* are inactivated in unstable hybrids and that CENH3 of *H. vulgare* can not compensate the lack of CENH3 of *H. bulbosum*. Against our initial expectation the expression analysis showed that all four parental CENH3s (Hvα and βCENH3 and Hbα and βCENH3) undergo expression in unstable hybrids similar to stable hybrids. On the other hand our analysis demonstrated that in stable *H. vulgare* x *H. bulbosum* hybrids CENH3 protein of *H. vulgare* is able to target the centromeres of *H. bulbosum* as well. Thus, uniparental reduction of CENH3 suggests that either the stability of already incorporated CENH3 is reduced or the incorporation of newly translated CENH3 into the centromeres of *H. bulbosum* is compromised. Consequently, loss of CENH3 will result in a non-functional centromere.

We noticed in unstable hybrids a different degree of condensation between both parental chromosomes. Often the chromosomes of *H. bulbosum* were less condensed (Gernand et al., 2006) (Fig. 38). In agreement with our observation, (Bennett et al., 1976) reported that *H. bulbosum* requires more time for the completion of a cell cycle than *H. vulgare.* The growth rate of embryos in *H. vulgare* and *H. bulbosum* and their hybrids were measured by counting the cell number of each genotype in different ages (Bennett et al., 1976). In hybrid tissues, the rate is intermediate, but often much nearer to that of *H. vulgare* (Fig. 39).

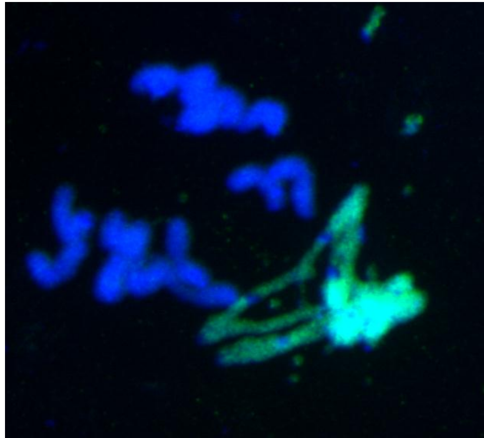


Fig. 38. Asynchrony of parental genomes in an unstable *H. vulgare* x *H. bulbosum* hybrid. A snake-like structure of *H. bulbosum* chromatin (in green) in anaphase indicates a delayed condensation process of *H. bulbosum* chromatin (Gernand et al., 2006b).

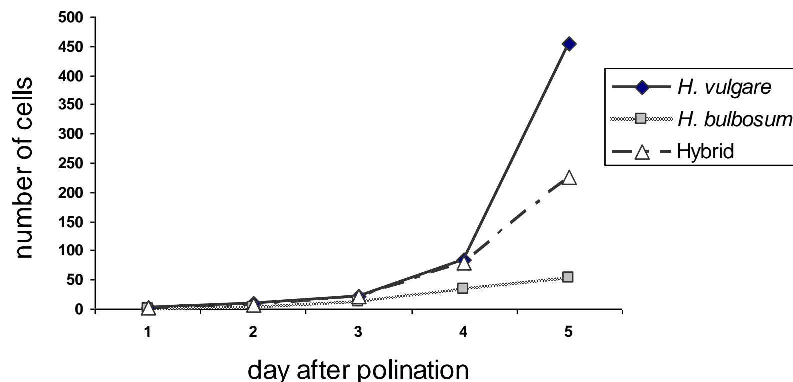


Fig. 39. Comparison of cell numbers in young embryos of *H. vulgare, H. bulbosum* and their hybrid 1 - 5 days after pollination (Bennett et al., 1976).

As the right time of centromeric histone H3 deposition is of functional importance, cell cycle asynchrony might interfere with the proper loading of CENH3 in unstable hybrids. Thus, the genome of *H. bulbosum* undergoes elimination although CENH3 transcripts and proteins are available. The centromeric chromatin of *H. bulbosum* is not 'ready' to incorporate CENH3 proteins and after a few cell divisions the parental CENH3 becomes diluted. As a consequence centromeres are getting inactive and finally chromosomes of *H. bulbosum* are

undergoing elimination. This, however does not exclude the possibility that other factors may also contribute to the inability to assemble active *H. bulbosum* centromeres in unstable hybrids. The possible mechanisms of chromosome elimination in unstable *H. vulgare* x *H. bulbosum* hybrids is summarized in figure 40.

Notably, defects in proteins involved in the loading of CENH3 in non-plant organisms result in abnormalities very similar to the CENH3 depletion itself. For instance, centromeric recruitment of newly synthesized CENH3 is rapidly abolished in any of the knocked down by RNAi of Mis18α/β or M18BP1 in human (Fujita et al., 2007), or depletion of KLN2 in *C. elegans* prevent localization of the CENH3 to chromatin (Maddox et al., 2007). Depletion of the Holliday Junction Recognition Protein (HJURP), by tree cell cycles, caused substantially reduction in the CENH3 level, micronuclei formation and chromosome missegregation consistent with failure to load new CENH3 and/or loss from centromeres (Foltz et al., 2009; Shuaib et al., 2010). It was reported that HJURP binds to the CATD domain of CENH3 via its N-terminal region (Foltz et al., 2009; Shuaib et al., 2010). In addition, RSF-1 depletion induced loss of centromeric CENH3, cell cycle delay and chromosome misalignment (Perpelescu et al., 2009). Also, members of the human Mis12 complex, although not constitutively centromeric, can influence CENH3 centromere occupancy (Kline et al., 2006). Proteins involved in the same centromeric function in plants are unknown.

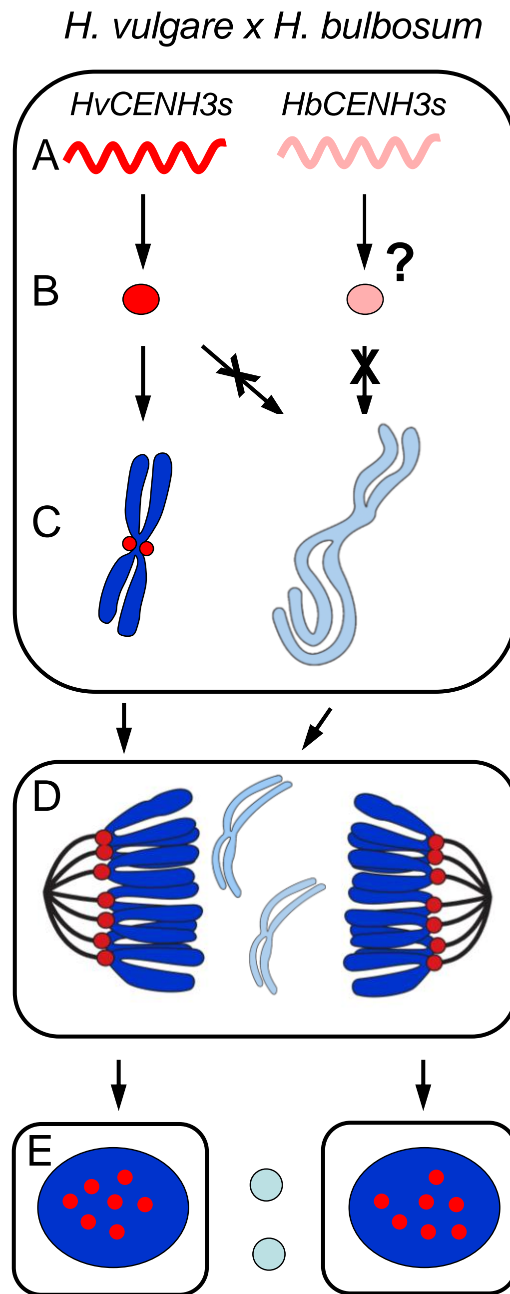


Fig. 40. Model on the role of CENH3 in the process of uniparental chromosome elimination in unstable *H. vulgare* x *H. bulbosum* hybrid embryos. (A) Both parental CENH3s are expressed, (B) translation of HvCENH3 occurs, but it is unknown whether translation of HbCENH3 occurs too. (C) Loading of CENH3 into the centromeres of *H. vulgare* but not of *H. bulbosum* due to cell cycle asynchrony of the two parental genomes. (D) Only centromeres of *H. vulgare* are undergoing a correct mitosis. In contrast, *H. bulbosum* chromosomes are lagging and will form (E) subsequently micronuclei. Finally micronucleated *H. bulbosum* chromatin will be degraded and the embryo will be haploid.

5.4. The influence of temperature on the process of chromosome elimination

Chromosome elimination also depends on the temperature after fertilization (Pickering and Morgan, 1985; Thomas and Pickering, 1983). A temperature above 18°C during the early stages of embryo growth can promote chromosome elimination. How might the temperature influence the process of chromosome elimination? Temperature-mediated changes in nucleosome composition via chaperon deposition of histone variants are recently demonstrated for plants (Kumar and Wigge, 2010). Chaperons share the ability to recognize and bind nascent and unfolded proteins, thus preventing aggregation and facilitating correct protein folding (Frydman, 2001). They function also in protein stabilization and play a key role in cell-cycle control too (Wang et al., 2004). If temperature-mediated changes in centromeric nucleosome assembly exists the temperature effect on the process of uniparental chromosomes could be explained. However, it is unknown whether chaperons, involved in CENH3-loading are temperature sensitive. Further, as temperature influence the cell cycle time (Grif et al., 2002) a low temperature probably may speed down the cell division in *H. vulgare* but not of *H. bulbosum.* As a consequence the cell cycle asynchrony of the two parental genomes is reduced which could increase the hybrid stability.

5.5. Cross-species incorporation of HvCENH3 in stable hybrids, substitution and addition lines

Immunolabeling of stable *H. vulgare* x *H. bulbosum* hybrids with anti-HvCENH3 antibodies demonstrated that both CENH3 variants of *H. vulgare* are able to incorporate into the centromeres of *H. bulbosum.* But, as anti-HbCENH3 antibodies were not available it was impossible to determine whether HbCENH3 proteins are targeting *H. vulgare* and/or *H. bulbosum* centromeres in stable *H. vulgare* x *H. bulbosum* hybrids.

A different situation was observed for the more distantly related species combination. In wheat-barley 1H + 6H addition line up to 46 HvαCENH3 immunosignals were detected. In contrast, no centromeric incorporation of HvβCENH3 was found while RT-PCR demonstrated the transcription activity of both *H. vulgare* CENH3s. Thus, despite differences in centromeric DNA composition between wheat and barley, no species-specific incorporation of CENH3 occurs if CENH3 of both parents coexist. However, not all parental CENH3 variants are necessarily incorporated, because HvβCENH3 was not found in centromere of wheat.

Probably as the CENH3s of wheat are more sequence similar to the αCENH3-variant of *H. vulgare* (Evtushenko and Houben, personal communication) a preferential incorporation of HvαCENH3 occurred into the centromeres of wheat and barley. As the CAT domain of CENH3 is required for the target of the CENH3 to the centromere (Vermaak et al., 2002), it is possible that the CAT domain of HvαCENH3 has a higher affinity to the centromeres of wheat than HvβCENH3 does.

In wheat – barley 1H + 6H addition line, CENH3 of barley requires most probably the CENH3-loading machinery of wheat. CENH3 chaperons in other organism interact with CENH3 via its CAT domain. If we consider similar CENH3 loading machinery in plants, then, due to higher sequence differences between CENH3s of wheat and βCENH3 of barley (Fig. 41), no incorporation of HvβCENH3 occurs. While HvαCENH3 can use the CENH3-loading machinery of wheat due to the higher similarity to wheat CENH3s and target the centromeres of wheat and barley.

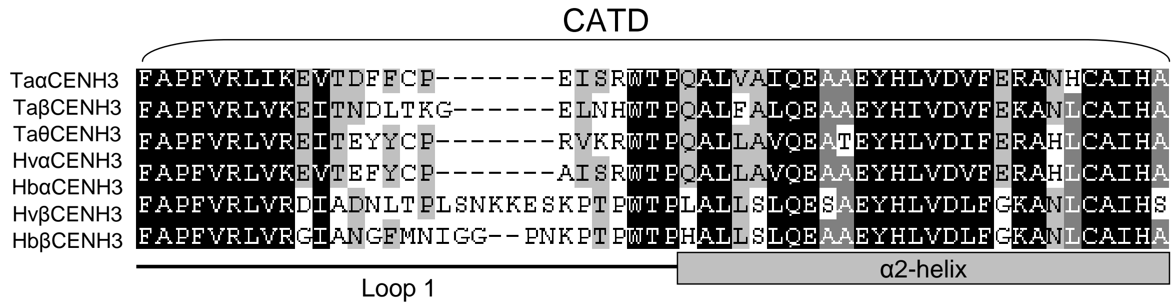


Fig. 41. Comparison of the CAT domains (Loop1 and α2-helix) of α and β types of *H. vulgare* CENH3s (HvCENH3), *H. bulbosum* CENH3s (HbCENH3) and *T. aestivum* CENH3s (TaCENH3). Note the higher sequence similarity between CATD of HvαCENH3, HbαCENH3 and TaCENH3s than of HvβCENH3.

The analysis of the *H. vulgare*-*H. bulbosum* 7H substitution line demonstrated that CENH3 of *H. bulbosum* is not required for cross-species HvCENH3 incorporation. Further α and ßCENH3 of *H. vulgar*e can functionally compensate the missing CENH3 of *H. bulbosum*. Hence, for cross-species incorporation of CENH3, an 'original' CENH3 is not required.

Our analysis of stable hybrids and of chromosome addition and of substitution lines demonstrates that cross-species incorporation of CENH3 occurs. Hence, after sexual hybridisation CENH3 of one parent can rescue the centromere function of the other parental species involved, despite centromere-sequence differences. This finding is strongly supported by the availability of a large number of chromosome addition lines. Even remotely related species as such as oat and maize can be sexually hybridized to produce fertile partial hybrids (Kynast et al., 2001). In this species combination, CENH3 of oat compensates the missing CENH3 of maize as silencing of maize CENH3 in the genetic background of oat in oat-maize chromosome addition lines occurred (Jin et al., 2004). Similarly, the CENH3 protein from *A. thaliana* can be detected at the centromeres of all chromosomes of allotetraploid *A. thaliana* x *A. arenosa* hybrid *A. suecica* (Talbert et al., 2002).

Our observation based on sexually generated hybrids supports previous cross-species CENH3 incorporation experiments performed in transgenic organisms (Moraes et al., 2010; Nagaki et al., 2010; Ravi et al., 2010), The authors reported that CENH3s of closely related species are able to target centromeres in alien species. For instance CENH3 of *Arabidopsis lyrata*, *A. arenosa*, *Capsella bursa-pastoris* and *Z. mays* target *A. thaliana* centromeres (Moraes et al., 2010) and *A. thaliana* CENH3 targets centromere of tobacco (Nagaki et al., 2010).

6. Outlook

6.1. Characterization of the function and two CENH3 variants

Existence of more than one CENH3 is very rare in diploid organisms. Thus, it would be of interest to analyse the function of multiple CENH3 variants and to study whether all CENH3 variants are required for a functional centromere. This could be studied by the generation of mutant barley lines with modified α or βCENH3 activity using the TILLING (Targeting Induced Local Lesions IN Genome) approach.

6.2. Interaction analysis of CENH3 variants

Our study shows that α and βCENH3 proteins of *H. vulgare* intermingle in meiotic, mitotic and interphase centromeres. .But, it is unkown whether they are deposited to the same or different centromeric nucleosomes and whether they are interacting with similar or different DNA sequences. Performing chromatin immunopercipitation-sequencing (ChIP-seq) experiments will help to understand whether each CENH3 variant interacts with same or different centromeric DNA sequences. To test whether α- and βCENH3 are directly interacting and therefore forming octameric nucleosomes tandem-immunopercipitation experiments on isolated mono nucleosomes of barely with HvαCENH3- and HvβCENH3-specific antibodies could be performed in future.

6.3. Investigation of CENH3 incorporation in allopolyploid species

It will be interesting to study how many different CENH3 variants are incorporated into centromeres of polyploid species. It will help to understand whether cross capability between species depends on the ability of centromeres to incorporate different parental CENH3 variants. For that immunostaining

experiments could be done on natural or synthetic plant hybrids using CENH3-variant-specific antibodies.

6.3. Study the CENH3 loading machinery in plants

CENH3 priming, loading and maintaining factors are studied in human, yeast and fruit fly. It would be interesting to determine their orthologues in plants as well. As CENH3 has a different incorporation time in plants it was suggested that they may have different incorporation machinery as well. This information will help to investigate whether CENH3 loading factors have a role in chromosome elimination. Does each CENH3 variant employs its own set of assembly factors in hybrids? And, are CENH3 chaperons temperature dependent like other histone chaperons?

7. Summary

Uniparental chromosome elimination occurs in some interspecific hybrids. Although this process has been exploited to produce doubled haploids, the mechanism behind it is not well understood.

The aim of this project was to investigate the processes of selective chromosome elimination of one parental genome during the development of hybrid embryos and to establish whether the highly conserved centromeric histone H3 protein (CENH3) regulates chromosome elimination. *Hordeum vulgare* x *H. bulbosum* crosses were used as a model, because in this species combination the degree of chromosome elimination/retention is influenced by the genotype of both parents as well as environmental conditions after fertilization.

In unstable *H. vulgare* x *H. bulbosum* hybrid embryos lagging *H. bulbosum* chromosomes and subsequently formed micronuclei were CENH3-negative. Seven weak and seven strong CENH3-specific signals in hybrid nuclei were observed indicating an important role of CENH3 in chromosome elimination.

Cloning of the CENH3 genes of *H. vulgare* (cv. 'Emir') and of *H. bulbosum* revealed two CENH3 variants in both species. Different wheat/barley addition lines and barley/*H. bulbosum* substitution lines were used for mapping of CENH3 genes. Result shows that in both species αCENH3 and βCENH3 are encoded by chromosome 1H and 6H, respectively.

Loading analysis of CENH3 using specific anti-α and βCENH3 antibodies revealed that both CENH3 variants incorporate to the centromeres at G2 phase.
α and βCENH3 proteins of *H. vulgare* intermingle in meiotic, mitotic and interphase centromeres. Thus, both CENH3 variants are equally involved in the formation of mitotic and meiotic barley centromeres.

Transcription analysis of CENH3s in stable and unstable hybrid embryos indicates that CENH3 genes of both parents are equally active in stable and unstable hybrid embryos. Hence, either mRNA of *H. bulbosum* CENH3s is not translated to an active protein or CENH3 incorporation is impaired, probably due to asynchrony of the two parental genomes rather than uniparental silencing of CENH3 genes. As a consequence centromeres of *H. bulbosum* are inactive in unstable hybrids.

Hvα and βCENH3 proteins both can incorporate into *H. bulbosum* centromeres in stable *H. vulgare* x *H. bulbosum* hybrids and also into centromeres of *H. bulbosum* chromosomes stably added to barley in *H. vulgare*/*H. bulbosum* substitution lines. In wheat/barley addition lines, HvαCENH3 is able to target wheat centromere. In contrast, HvβCENH3 although transcribed does not undergo centromere incorporation. Thus in stable species combinations cross-species incorporation of CENH3 occurs despite centromere-sequence differences, but not all CENH3 proteins variants get incorporated into centromeres if multiple CENH3 genes are present in species combinations.

8. Zusammenfassung

Uniparentales Eliminieren von Chromosomen existiert in einigen interspezifischen Hybriden. Obgleich dieser Vorgang bei der Herstellung doppelt-haploider Pflanzen genutzt wird, ist dieser Prozess erst wenig verstanden.

Projektziel war es zu erkunden, inwieweit die konservierte Histon-H3 Variante CENH3 den Vorgang der selektiven Chromosomenelimination reguliert. *Hordeum vulgare* x *H. bulbosum* Kreuzungen wurden als Model genutzt, da in dieser Kombination der Chromosomeneliminationsvorgang sowohl vom Genotyp aber auch von Umweltbedingungen abhängig ist.

In instabilen *H. vulgare* x *H. bulbosum* Kreuzungsembryonen sind die Chromosomen von *H. bulbosum* und die daraus entstandenen Mikrokerne CENH3-negativ. Der Nachweis von sieben stark und sieben schwach markierten CENH3-Signalen in einigen Zellkernen von instabilen Kreuzungsembryonen unterstützt die Annahme, dass CENH3 im Prozess der uniparentalen Chromosomeneliminierung involviert ist.

H. vulgare (cv. 'Emir') und *H. bulbosum* codiert je zwei unterschiedliche CENH3 Genvarianten (αCENH3 und βCENH3). Unterschiedliche Weizen/Gersten-Additionslinien and Gersten/*H. bulbosum*-Substitutionslinien wurden für die Kartierung der CENH3 Gene eingesetzt. αCENH3 wird von Chromosom 1H und βCENH3 wird von Chromosom 6H codiert.

Die Genaktivität von CENH3s in stabilen und instabilen Kreuzungsembryonen ist ähnlich. Damit kann gefolgert werden, dass entweder CENH3-Transkripte nicht in funktionell Proteine translatiert werden, oder der zentromerische Einbau von

CENH3 nicht gewährleistet ist. Grund dafür, könnte die ungleichschnelle Zellteilung beider elterlicher Genome sein.

Hvα and βCENH3 Proteine können in die Zentromere von *H. bulbosum* Chromosomen, in stabilen *H. vulgare* x *H. bulbosum* Hybriden und in *H. vulgare*/*H. bulbosum*-Substitutionslinien eingebaut werden. HvαCENH3 in Weizen/Gersten-Additionsliniein wird in die Zentromere von Gerste und Weizen eingebaut. Dagegen werden HvβCENH3 Proteine in dieser Kombination nicht eingebaut trotz Transkription. Damit ist der Einbau von Fremd-CENH3 in stabilen Artkombinationen Zentromersequenz-unabhängig, aber nicht alle CENH3 Varianten werden in Zentromere eingebaut.

9. Literature

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10. Supplementary material

Appendix Table 1. A pair-wise comparison of amino acid identity and similarity of *H. vulgare*, *H. bulbosum* and *H. marinum* CENH3s in percent.
* Note, the N-terminal region of *H. marinum* βCENH3 is unknown.

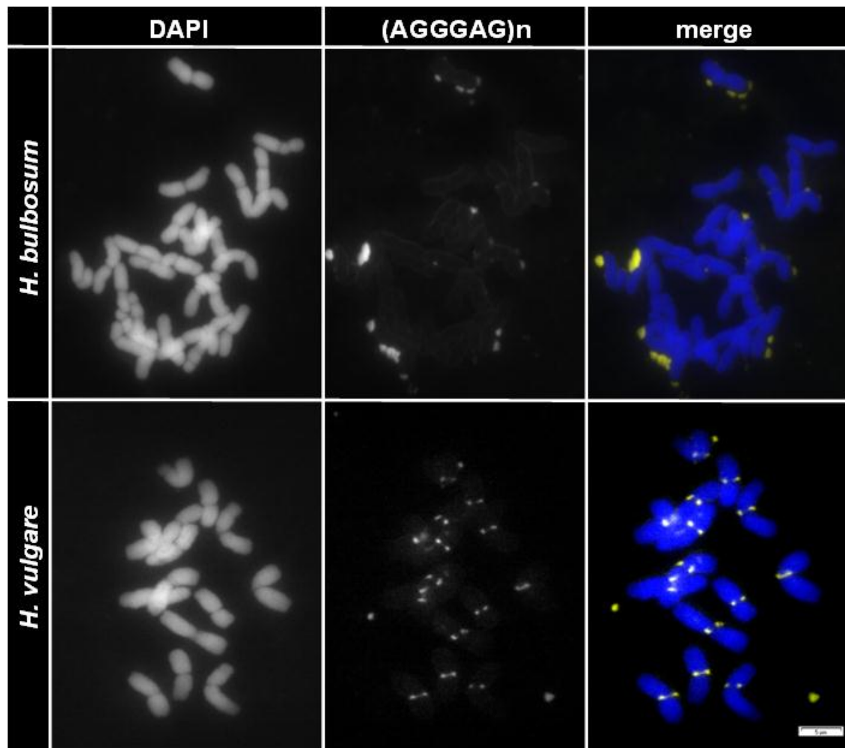
		H. vulgare		*H. bulbosum*		*H. marinum*	
		αCENH3	βCENH3	αCENH3	βCENH3	αCENH3	βCENH3*
H. vulgare	αCENH3	-	54.1	91.2	57.6	76.8	70.9
	βCENH3	-	-	52.9	71.4	51.8	66
H. bulbosum	αCENH3	-	-	-	54	77.6	73.3
	βCENH3	-	-	-	-	58.8	67.3
H. marinum	αCENH3	-	-	-	-	-	76.3
	βCENH3	-	-	-	-	-	-

Appendix Fig. 1. Nucleic acid sequence alignment of *H. vulgare* (HvαCENH3), *H. bulbosum* (HbαCENH3), *H. marinum* (HmαCENH3), *H. vulgare* (HvβCENH3), *H. bulbosum* (HbβCENH3) and of *H. marinum* (Hm βCENH3). Residues that are completely conserved in sequence are shown in white on a black background, those that are ≥80% conserved are shown in white on grey background and ≥60% identical residues are shown in black in grey background. The gaps are shown as dashes. CENH3s are more variable in N-terminal regions and more conserved in C-terminal.

```
          v10       v20       v30       v40       v50       v60       v70       v80       v90       v100      v110      v120      v130      v140      v150      v160      v170
GTGGCACTGCGGGAGATCAGGAAGTACCACAAGTCCACCGAACTGCTCATCCCCTTTGCGCCCTTCGTCCGCCTGGTGAGTGCGGGTTGGGTACCCCGTACCCGTGTCCCCCCTGAGCCCCATCTGCTCTTCCTGTCCTCAGATGTGAATCTCCATCACCGATTCAGGAT
GTGGC CTGCGGGAGATC GGAAGTACC CAAGTCCACCGAACTGCTCATCCCCTTTGCGCCCTTCGTCCGCCTGGT
GTGGCGCTGCGGGAGATCCGGAAGTACCGCAAGTCCACCGAACTGCTCATCCCCTTTGCGCCCTTCGTCCGCCTGGT--------------------------------------------------------------------------------------------
  ^120      ^130      ^140      ^150      ^160      ^170      ^180      ^190
          v180      v190      v200      v210      v220      v230      v240      v250      v260      v270      v280      v290      v300      v310      v320      v330
CTGCTCTTCCTGTCCTCAGATGTGAATCTCCATCACCGATTCAGGATGCGGAGACTGTGTGTGATGGAAACTTGCTCGTCTTGTGCTGATGGTAAATTTGCAGGTTAGGGGAATCGCCAACGGCTTCATGAATATCGGGGGGTCCAACAAGCCGACGCCAT
                                                                                                         TAGGGGAATCGCCAACGGCTTCATGAATATCGGGGGG CCAACAAGCCGACGCCAT
---------------------------------------------------------------------------------------------------------TAGGGGAATCGCCAACGGCTTCATGAATATCGGGGGGCCCAACAAGCCGACGCCAT
                                                                                                              ^200      ^210      ^220      ^230      ^240      ^250

          v10       v20       v30       v40       v50       v60       v70       v80       v90       v100      v110      v120      v130      v140      v150      v160      v170
GTGGCACTGCGGGAGATCAGGAAGTACCGCAAGTCCACCGAACTGCTCATCCCCTTTGCGCCCTTCGTCCGCCTGGTGAGTGCGGGTTGGGTACCCCGTACCCGTGTCCCCCCTGAGCCCCATCTGCTCTTCCTGTCCTCAGATGTGAATCTCCATCACCGATTCAGGAT
GTGGC CTGCGGGAGATC GGAAGTACCGCAAGTCCACCGAACTGCTCATCCCCTTTGCGCCCTTCGTCCGCCTGGT
GTGGCGCTGCGGGAGATCCGGAAGTACCGCAAGTCCACCGAACTGCTCATCCCCTTTGCGCCCTTCGTCCGCCTGGT--------------------------------------------------------------------------------------------
  ^120      ^130      ^140      ^150      ^160      ^170      ^180      ^190
          v180      v190      v200      v210      v220      v230      v240      v250      v260      v270      v280
GCGGAGACTGTGTGTGATGGAAACTTGCTCGTCTTGTGCTGATGGTAAATTTGCAGGTTAGGGGAATCGCCAACGGCTTCATGAATATCGGGGGGTCCAACAAGCCGACGCCAT
                                                           TAGGGGAATCGCCAACGGCTTCATGAATATCGGGGGG CCAACAAGCCGACGCCAT
-----------------------------------------------------------TAGGGGAATCGCCAACGGCTTCATGAATATCGGGGGGCCCAACAAGCCGACGCCAT
                                                                ^200      ^210      ^220      ^230      ^240      ^250
```

Appendix Fig. 2. A 47 bp region duplication within the forth intron of HbβCENH3.



Appendix Fig. 3. Confirming the specificity of the centromeric repeat (AGGGAG)n that labels specifically *H. vulgare* centromeres but not *H. bulbosum* centromeres. Centromeric specific signals are in yellow. Scale bar presents 5 µm.

11. Publications and Proceedings related to the submitted thesis

Sanei, M., R. Pickering, J. Fuchs, A.M. Banaei Moghaddam, A. Dziurlikowska & A. Houben. (2010). Interspecific hybrids of *Hordeum marinum* ssp. *marinum* x *H. bulbosum* are mitotically stable and reveal no gross alterations in chromatin properties. Cytogenetic and Genome Research 129: 110-116.

Houben, A., **Sanei, M**., Pickering, R. (2010). Barley doubled-haploid production by uniparental chromosome elimination. PCTOC: Plant Cell Tissue Organ Culture. 104:321–327.

Sanei, M., Pickering, R., Kumke, K., Nasuda, S., Houben, A. (2011). Loss of CENH3 from centromeres proceeds uniparental chromosome elimination in interspecific barley hybrids. PNAS USA. (in press)

Poster and talks

Sanei, M., Pickering, R., Houben, A. (2008). Analysis of uniparental elimination of chromosome in wide crosses, 4th Plant Science Student Conference (PSSC 2008), 4- 6 June, Leibniz Institute for Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany (Presented as poster).

Sanei. M., Pickering, R., Houben, A. (2009). Analysis of uniparental elimination of chromosome in wide crosses, 5th Plant Science Student Conference (PSSC 2009), 23- 26 June, Leibniz Institute of Plant Biochemistry (IPB), Halle, Germany (Talk given by. M. Sanei).

Sanei, M., Pickering, R., Nasuda, S., Houben, A. (2009). Analysis of uniparental elimination of chromosome in wide crosses. Annual Conference of the German Genetics Society GfG, 16-19 September 2009, Cologne, Germany, (Presented asposter).

Sanei, M., Pickering, R., Nasuda, S., Houben, A. (2010). Analysis of uniparental elimination of chromosome in wide crosses. 6th Plant Science Student Conference 2010 (PSSC 2010), 15-18 June, Leibniz Institute for Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany (Talk given by. M. Sanei).

Sanei, M., Pickering, R., Nasuda, S., Houben, A. (2010). Analysis of uniparental elimination of chromosome in wide crosses. Society for

Experimental Biology (SEB) annual main meeting, 30 June- 3 July 2010, Prague, Czech Republic (Presented as poster).

Sanei, M., Pickering, R., Nasuda, S., Houben, A. (2010). Analysis of uniparental elimination of chromosome in wide crosses. 10th Gatersleben Research Conference (GRCX), 22 - 24 November 2010, Gatersleben & Quedlinburg, Germany (Presented as poster).

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12. Eidesstattliche Erklärung

Diese Promotionsschrift wurde selbständig und ohne fremde Hilfe verfasst. Andere als die angegebenen Quellen und Hilfsmittel wurden nicht benutzt. Wörtlich oder inhaltlich übernommene Stellen wurden als Zitate gekennzeichnet.

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12- Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit von mir bisher weder der Naturwissenschaftliche Fakultät III der Martin-Luther- Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.

Ich erkläre ferner, dass ich diese Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel und Literatur angefertigt habe.

Gatersleben, den ....

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Researches

- Analysis of uniparental elimination of chromosome in wide crosses (Ph.D. thesis).
- Karyotype Study in Iranian *Crocus* Species (M.Sc thesis).
- Investigation of the effect of two plant growth regulators (CCC and PBZ) on environmental stress-tolerance and morphological characteristics of pistachio seedlings (B.Sc thesis)

Job Experiences

- Working on Ph.D. thesis as an employee at the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany (Oct.2007- 2011).
- Working as an M.Sc student doing my thesis in Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Tehran, Iran (Sept.2004-May 2006)

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