Restructuring of holocentric centromeres during meiosis in the plant

*Rhynchospora pubera*

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Abstract:

Centromeres are responsible for the correct segregation of chromosomes during mitosis and meiosis. Holocentric chromosomes, characterized by multiple centromere units along each chromatid, have particular adaptations to ensure regular disjunction during meiosis. Here we show by detecting CENH3, CENP-C, tubulin and centromeric repeats that holocentromeres may be organized differently in mitosis and meiosis of *Rhynchospora pubera*. Contrasting to the mitotic linear holocentromere organization, meiotic centromeres show several clusters of centromere units (cluster-holocentromeres) during meiosis I. They accumulate along the poleward surface of bivalents where spindle fibers perpendicularly attach. During meiosis II, the cluster-holocentromeres are mostly present in the mid-region of each chromatid. A linear holocentromere organization is restored after meiosis during pollen mitosis. Thus, a not yet described case of a cluster-holocentromere organization, showing a clear centromere restructuration between mitosis and meiosis, was identified in a holocentric organism.
Key words: holocentric chromosomes, CENH3, CENP-C, centromere structure/organization, inverted meiosis

Introduction

The centromere is the chromosome site responsible for spindle fiber attachment and faithful chromosome segregation during mitosis and meiosis. In general, every eukaryotic chromosome has a centromere on which the kinetochore complex assembles (CLEVELAND et al. 2003; BURRACK AND BERMAN 2012). In most eukaryotes, centromeric nucleosomes contain CENH3 (also known as CENP-A, a histone H3 variant that replaces canonical H3 at the centromere), and usually spans several hundred kilobase-pairs (kb) often in association with centromere-specific repeats (STEINER AND HENIKOFF 2015).

Centromere organization and dynamics vary between mitosis and meiosis (DURO AND MARSTON 2015; OHKURA 2015). During mitosis, sister-chromatids are held together by centromere cohesion until metaphase. Simultaneous with the disruption of cohesion, sister-chromatids are pulled to opposite poles during anaphase. In contrast, during meiosis, sister centromere cohesion is ensured until metaphase II (ISHIGURO AND WATANABE 2007). The stepwise regulation of cohesion release during meiosis I and II is well-studied in organisms with one primary constriction per chromosome (monocentric), ensuring the segregation of homologs at meiosis I followed by the segregation of sister chromatids at meiosis II (DURO AND MARSTON 2015).

Contrary to monocentrics, the centromeres of holocentric chromosomes are distributed almost over the entire chromosome length and cohesion occurs along the
entire associated sister chromatids (MADDOX et al. 2004). Although this does not imply much difference during mitotic divisions, the presence of a holokinetic centromere (holocentromere) imposes obstacles to the dynamics of chromosome segregation in meiosis. Due to their alternative chromosome organization, species with holocentric chromosomes cannot perform the two-step cohesion loss during meiosis typical for monocentric species that requires the distinction between chromosome arms and sister centromeres (HAARHUIS et al. 2014). In addition, the extended holocentric kinetochore increases the risk of a stable attachment to microtubules from both poles of the spindle (merotelic attachment), and hence an aberrant segregation of chromosomes may occur. As adaptation, species with holocentric chromosomes have evolved different solutions during meiosis, such as a restricted kinetochore activity, ensuring canonical meiosis order, and “inverted meiosis”, where a reverse order of sister chromatid and homologue separation occurs (see below) [reviewed in VIERA et al. (2009); CUACOS et al. (2015)].

In the nematode *C. elegans*, the chromosomes form a single chiasma per bivalent at one of their termini that has the capacity to form crossovers (COs). The crossover location triggers the redistribution of proteins along the bivalent axis. Kinetochore components uniformly coat each half bivalent but are excluded from the so-called mid-bivalent region where CO occur (ALBERTSON et al. 1997; MARTINEZ-PEREZ et al. 2008). Although there are differences between male and female meiosis in regard to microtubule organization and attachment (SHAKES et al. 2009; WIGNALL AND VILLENEUVE 2009; DUMONT et al. 2010), in both cases, one pair of sister chromatids faces one spindle pole and the other pair belonging to the second homolog faces the opposite pole. Finally, the sister chromatids remain attached via one chromosome
end and become separated during the second meiotic division (ALBERTSON AND THOMSON 1993; MARTINEZ-PEREZ et al. 2008; DUMONT et al. 2010).

Meiotic adaptations are also observed in other holocentric organisms such as in *Heteroptera* (HUGHES-SCHRADER AND SCHRADER 1961; PEREZ et al. 2000; VIERA et al. 2009) and *Parascaris* species (PIMPINELLI AND GODAY 1989), where spindle fibers attach to a restricted kinetochore region at a single chromosome end of each homologue during meiosis I (telokinetic meiosis). Thus, this type of meiosis acts functionally as in monocentric species, since the homologs segregate to opposite poles already during meiosis I. Remarkably, during meiosis II the same telokinetic behavior is observed, although it seems to be random which one of the chromosomal termini acquires the kinetic activity in both divisions (MELTERS et al. 2012). These findings indicate a high plasticity for the centromere/kinetochore structures during meiotic divisions in holocentric organisms.

The holocentric plant species *Rhynchospora pubera* and *Luzula elegans* evolved an alternative strategy to deal with meiosis. They are characterized by showing the so-called “inverted meiosis” (CABRAL et al. 2014; HECKMANN et al. 2014), which means that sister chromatids segregate already at anaphase I, while the segregation of homologs is postponed to meiosis II (also called post-reductional meiosis). They also display individual chromatids at prophase II, indicating the complete loss of sister chromatid cohesion during meiosis I. However, meiosis is not truly inverted in these species; instead terminal chiasmata result in the exchange of some genetic material between homologous non-sister chromatids. Therefore, the segregating sister chromatids in meiosis I (MI) still consist of a part of homologous non-sister chromatids. Furthermore, in contrast to the restriction of the kinetochore activity
found in other holocentric species, *L. elegans* chromosomes show their
holocentromere structure and activity also throughout meiosis. They interact
individually and bi-orientated with the meiotic spindle. This results in the separation of
partially recombined sister chromatids already during meiosis I. To ensure a faithful
haploidization, the homologous non-sister chromatids remain linked at their termini
by chromatin threads after metaphase I until metaphase II, and separate at anaphase
II, leading to a post-reductional meiosis. Thus, an inverted sequence of meiotic sister
chromatid separation occurs (HECKMANN et al. 2014).

Similarly, in the Cyperaceae species *Rhynchospora pubera*, multiple spindle fibers
amphitetrically attach to the sister chromatids during meiosis I (GUERRA et al. 2010;
CABRAL et al. 2014). In mitosis, the chromosomes exhibit a linear holocentromere
organization comprising CENH3-containing centromere units enriched in centromeric
tandem repeats (named Tyba) and centromeric retroelements. In interphase, the
holocentromeres dissociate and form multiple individual centromere units. During
chromosome condensation towards mitotic metaphase, the centromeric units rejoin
and form a linear distinct longitudinal centromere within a groove to ensure faithful
chromosome segregation (MARQUES et al. 2015).

In contrast to mitotic chromosomes, where the (peri)centromeric histone marker
H2AThr120ph is also organized in a linear manner, a dispersed distribution was
found in meiotic chromosomes of *R. pubera*. In addition, multiple CENH3 patches
enhanced at the poleward chromosome surface of highly condensed metaphase I
bivalents were reported (CABRAL et al. 2014). This suggests a deviating centromere
organization during meiosis of *R. pubera*. However, the lack of simultaneous CENH3
and tubulin localization in other meiotic stages, and the limited microscopic resolution
hampered a comprehensive characterization of the kinetic activity and centromere organization throughout the meiosis of this species.

In order to shed more light in the meiotic centromere organization of *Rhynchospora*, we labelled centromeric proteins (CENH3 and CENP-C), repeats (Tyba) and α-tubulin, and applied super-resolution microscopy to characterize the organization and dynamics of *R. pubera* holocentromeres throughout meiosis. We report that the holocentromere organization of *R. pubera* differs significantly between mitosis and meiosis, providing the identification of a not yet reported meiotic centromere organization among eukaryotes.

**Material and Methods**

**Plant material.** *Rhynchospora pubera* (Vahl) Boeckler plants were cultivated under humid conditions at the Experimental Garden of the Laboratory of Plant Cytogenetics and Evolution (Recife, Brazil) and in a greenhouse at the Leibniz Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany).

**Identification and validation of the CENP-C gene and generation of CENP-C antibodies.** The *CENP-C* gene was identified *in silico* by BLAST search from the transcriptome data of *R. pubera* (accession number PRJEB9645, http://www.ebi.ac.uk/ena/). For the validation of expression, semi-quantitative RT-PCR was performed with DNase treated total RNA isolated from root, leaf and anther tissue of *R. pubera* using the SpectrumTM plant total RNA kit (Sigma). The cDNA was synthesized from 1 µg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). PCR reactions were performed with the primer sequences: forward 5’-AATGACTTCACCCTCACCCG-3’ and reverse 5’-
CCTTCTTGAGGTGCTAGTGC-3’. Primers for the constitutively expressed GAPDH
gene (Banaei-Moghaddam et al. 2013), GAPDH-F
CAATGATAGCTGCACCACCAACTG and GAPDH-R
CTAGCTGCCCTCCACCTCTCCA, were used as control for applying equal
amounts of gDNA and cDNA. The amplified fragments were cloned into the
of 10 randomly selected clones revealed only one CENP-C variant (GenBank,
accession number KU516997).

The peptide VRVKSFMSDEHADLIAKLAK was used to generate R. pubera CENP-C-
specific (RpCENP-C) polyclonal antibodies. Peptide synthesis, immunization of
rabbits and peptide affinity purification of antisera was performed by LifeTein
(http://www.lifetein.com).

**Phylogenetic analysis of plant CENP-C sequences.**
Reference IDs for all CENP-C sequences used in this study are available in Table
S2. A multiple alignment of protein sequences encoding the entire CENP-C
sequences was generated using MAFFT (Kato and Standley 2013) and refined
manually. Evolutionary analyses were conducted with IQ-TREE (Nguyen et al. 2015)
using ultrafast bootstrap (Minh et al. 2013). Phylogenetic history was inferred using
the Maximum Likelihood method using the Best-fit model: JTT+I+G4 acquired
automatically with IQ-TREE. The analysis involved 30 protein sequences. The
alignments and trees are available through the iPlant Data Store and can be
accessed via iPlant Discovery Environment or at
http://de.iplantcollaborative.org/dl/d/C34A1998-A409-4E52-A732-
2FCD8C906E53/RpCENPC.rar.
**Immunostaining of somatic and meiotic cells.** Immunostaining for CENH3 and CENP-C was performed as described in CABRAL et al. (2014) with some modifications. Anthers were fixed in ice-cold 4% paraformaldehyde in 1×PBS buffer pH 7.5 (1.3M NaCl, 70mM Na₂HPO₄, 30mM NaH₂PO₄) for 1 h 30 min and squashed in a drop of the same buffer. Alternatively, anthers were treated with colchicine 0.05% for 24h at 10 °C and fixed as above. Tapetum cells of young anthers were used for the preparation of mitotic cells. Then, the slides were washed in 1×PBS and blocked with 3% BSA for 30 min at 37 °C. The antibodies used were rabbit anti-RpCENH3 (MARQUES et al. 2015) directly labeled with FITC, and rabbit anti-RpCENP-C, both diluted 1:500 in 1% BSA in 1×PBS. The detection of anti-RpCENP-C was done with goat anti-rabbit-Cy3 (Sigma, #F9887), diluted 1:200 in 1×PBS containing 1% BSA. The slides were counterstained with 2 μg/ml 4’6-diamidino-2-phenylindole (DAPI) in Vectashield H-1000.

For the simultaneous detection of CENH3 and tubulin, the anthers were fixed in methanol:acetic acid (3:1) for 2 to 24 h. Then, the anthers were rinsed three times in 1×PBS for 5 min, and the pollen mother cells were squeezed out from the anthers and squashed in a drop of 1×PBS. The coverslips were removed after freezing in liquid nitrogen. Then, the material was washed in 1×PBS and immersed in 1× citric buffer for 1 min in a microwave at 800 W. Afterwards, the slides were immediately washed in 1×PBS. The immunostaining procedure was conducted as described above. The CENH3 antibodies were detected by Cy3 or Alexa488 goat anti-rabbit antibodies. Mouse anti-α-tubulin antibodies (Sigma, #T5168) were diluted 1:50 in 1×PBS containing 1% BSA and detected with Alexa488 or Cy5 goat anti-mouse antibodies (ThermoFisher, #A-11001) diluted 1:100 in the same buffer.
**CENH3 fluorescence measurements**

Comparative CENH3 fluorescence signal intensity measurements of degenerative and functional cells in pseudomonads were performed using ImageJ 1.48s (http://imagej.nih.gov/ij). For measurements, we used the previously described formula (GAVET and PINES 2010), as follows:

Whole-Cell Signal = Sum of the intensity of the pixels for one cell; Background signal = average signal per pixel for a region selected just beside the cell; Whole-cell signal corrected = whole-cell signal - (number of pixels for the selected cell × background).

**Fluorescence in situ hybridization (FISH).** The centromere-specific repeat Tyba was detected with directly labelled 5′-Cy3 oligonucleotides (Tyba1: ATTGGATTATACATGGTAATTACGCATATAAAGTGCAAATAATGCAATTC; Tyba2: ACAGATTCTGAGTATATTTGAGCATTTCAAGCGATTTTGCGATT) (Eurofins MWG Operon, http://www.eurofinsdna.com). FISH after immunostaining was performed as described by ISHII et al. (2015).

**Widefield and super-resolution fluorescence microscopy.** Widefield fluorescence images were recorded using a Leica DM5500B microscope equipped with a Leica DFC FX camera and a deconvolution system. To analyze the substructures and spatial arrangement of immunosignals and chromatin beyond the classical Abbe/Raleigh limit (super-resolution), spatial Structured Illumination Microscopy (3D-SIM) was applied using a Plan-Apochromat 63×/1.4 oil objective of an Elyra PS.1 microscope system and the software ZEN (Carl Zeiss GmbH). The images were captured using 405, 488, 561 and 642 nm laser lines for excitation and the
appropriate emission filters, and merged using the ZEN software (WEISSHART et al. 2016). The Imaris 8.0 (Bitplane) software was used to measure the degree of colocalization between CENH3 and CENP-C. Briefly, after loading ZEN SIM image stacks the Imaris colocalization tool was applied. An automatic threshold defined by the point spread function (PSF) was calculated and used to establish a new colocalization channel originating from the CENH3 and CENP-C channels. This resulting channel contains the channel statistics including the degree of colocalization (in %) and the Pearson’s and Mander’s coefficients. Imaris 8.0 was also applied to produce 3D movies.

Results

By applying a specific antibody against R. pubera CENH3 we detected a chromosome-wide random distribution of CENH3 from early prophase I until diakinesis in R. pubera (Fig. 1A-B). At metaphase I multiple clustered CENH3 signals appeared (Fig. 1C), and 3D surface rendering of the whole chromatin confirmed the absence of a centromere groove during meiosis (Fig. 1B; Movie S1). These results strongly contrast to the linear holocentromere formation in mitosis, where the chromosomes exhibit a distinct longitudinal centromere groove (MARQUES et al. 2015) (Fig. 1D, Movie S2).
Fig. 1 Contrasting holocentromere formation between meiosis and mitosis of *R. pubera*. CENH3 localization at the chromosomes during (A) zygotene, (B) diakinesis, (C) metaphase I and (D) somatic metaphase. Arrowheads in D indicate mitosis-specific centromere grooves. Bar = 5 µm.

To confirm this contrasting centromere organization we used the inner kinetochore protein CENP-C as an additional centromere-marker. CENP-C is a key component of most eukaryotic centromeres and links the inner and outer (microtubule-binding)
components of the kinetochore (EARNSHAW 2015). It has been shown that CENP-C colocalizes to CENH3, thus defining active centromere chromatin (CARROLL et al. 2010; KATO et al. 2013; FALK et al. 2015). A single CENP-C candidate (RpCENP-C) was identified in an in silico analysis of the pollen mother cell transcriptome of R. pubera. The alignment of a RT-PCR generated 713 bp partial transcript with the CENP-C sequences of other species supported the correct identification (Fig. S1A). Phylogenetic analysis grouped RpCENP-C as a sister branch of Juncaceae, and both as sister branches to the Poaceae clade (Fig. S1B). Based on the identified sequence, RpCENP-C antibodies were generated.

During mitosis, R. pubera CENP-C and CENH3 specific centromeric signals were observed in interphase nuclei as dispersed dot-like structures not so well colocalized (Fig. 2A-B). A progressive colocalization of both centromere marks was observed during prophase and pro-metaphase when chromosomes displayed interrupted linear CENH3/CENP-C signals (Fig. 2C-D). Finally, at metaphase onset, chromosomes showed both CENP-C and CENH3 signals colocalized along the mitotic groove of all chromosomes (Fig. 2E; Movie S3). Based on ultrastructural analyses by super-resolution microscopy at a lateral resolution of ~140 nm the overlap between CENP-C and CENH3 signals was quantified. Compared to interphase, the degree of colocalization nearly doubled in prophase and further increased in metaphase (Table S1). This indicates the presence of CENP-C in addition to CENH3 at the centromeres of R. pubera at different mitotic stages and a progressive cell-cycle-dependent colocalization of both proteins.
Fig. 2 CENH3 and CENP-C distribution during the mitotic cell cycle of *R. pubera*, obtained from tapetum cells. (A-B) Interphase, (B) enlargement of A (squared), (C) prophase; (D) prometaphase and (E) metaphase. Colocalized CENH3 and CENP-C signals are visible in yellow in the merge images. Bar = 5 µm, except when indicated.

To validate the contrasting centromere organization observed on meiotic chromosomes, again we performed co-immunostaining with CENH3 and CENP-C.

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antibodies. From early prophase I until diakinesis, CENH3 and CENP-C are evident as partially colocalized dispersed dot-like signals all over the chromosomes (Fig. 3A-B; Movie S4). At metaphase I, the bivalents are arranged at the equatorial plate and both CENH3 and CENP-C cluster along the poleward surface of the chromatids (Fig. 3C and 3E; Movie S5). Similar as in somatic tissue, a clearly increased association between CENH3 and CENP-C was observed during meiosis compared to interphase (Table S1). At metaphase II, CENH3 and CENP-C are also highly clustered mostly occupying the mid-region of each chromatid (Fig. 3D). Hence, in contrast to the linear holocentromere organization observed during mitosis, a deviating assembly of centromere units occurs during meiosis, forming the so-called cluster-holocentromeres.
**Fig. 3** Distribution of CENH3 and CENP-C during different meiotic stages. (A) Diplotene, (B) diakinesis, (C) metaphase I and (D) metaphase II. (E) Metaphase I cell showing the colocalization of CENH3 and CENP-C in the cluster-holocentromeres. Overlapping signals are yellow in the merged images. Bar in A is equivalent to 5 µm for all images, except when indicated.

The mitotic holocentromeres of *R. pubera* are composed of centromeric tandem repeats called Tyba (cenDNA) (Marques et al., 2015). The colocalization of CENH3 and cenDNA is also evident throughout meiosis I and II (Fig. S2A-C). Thus, despite a different centromere organization, the DNA composition of the centromere units does not differ between meiosis and mitosis, and Tyba repeats can be used as additional makers for tracking the centromere organization during meiosis.

To check how and when the spindle fibers attach to the centromere units, the distribution of α-tubulin and CENH3/cenDNA were analyzed throughout meiosis. From early prophase I until diakinesis, no colocalization was found between spindle fibers and centromeres, which were scattered all over the chromosomes (Fig. 4A; Fig. 5A-B; Movie S6). At diakinesis, the bivalents are visible as typical rod- and ring-bivalents, corresponding to one and two chiasmata, respectively (Fig. 4A). At early metaphase I the bivalents are equatorially oriented and clustered CENH3/cenDNA signals are mostly enriched along the poleward surface of the bivalents, showing a bipolar orientation of the sister chromatids (Fig. 4B; Fig. 5C; Fig. S2D). At late metaphase I, the centromere units become less clustered and the sister cluster-holocentromeres colocalize with the spindle fibers from opposite poles (amphitelic attachment) (Fig. 4C; Fig. S2D; Movie S7). Univalents are often (3.5%) found in *R. pubera* (CABRAL et al. 2014) and they always show the same amphitelic attachment.
(Fig. 5D). At anaphase I, the sister cluster-holocentromeres are pulled by spindle fibers from opposite poles, resulting in the separation of sister chromatids (Fig. 4D; Fig. 5E). At this stage the spindle fibers are clearly colocalized with less clustered centromere units (Fig. 4E; Movie S8), likely a result of centromere tension. Chromatids migrate as single chromatids in both univalents and bivalents (Fig. 5E), supporting the early loss of sister chromatid cohesion and chiasmata resolution. At telophase I, the cluster-holocentromeres are mainly accumulated in the mid-region of each chromatid and show less colocalization with the spindle fibers (Fig. 4F). Thus, despite of the different centromere organization during meiosis I, the centromere units colocalize with the spindle fibers during meiosis.
Fig. 4 CENH3 and α-tubulin arrangement during meiosis. (A) Diakinesis, (B) early and (C) late metaphase I, (D) anaphase I, (E) enlargement of D (squared) and (F) telophase I. Interpretation models are illustrated at the last right column; sister chromatids are indicated by equal greyscales, while dark and light grey indicate
homologs. Putative crossovers are indicated by exchanged light and dark grey chromatin (arrowheads). While in (A) rod-bivalents have one chiasma, ring-bivalents have two of them. The dashed white and yellow lines indicate early sister chromatid cohesion loss and chiasmata resolution, respectively. Bar in F is equivalent to 5 µm for all images, except when indicated.

**Fig. 5** cenDNA (Tyba) and α-tubulin arrangement during meiosis I. (A-B) Detection of cenDNA during prophase I. (C-E) cenDNA and α-tubulin distribution on (C-D) metaphase I and (E) anaphase I. Inserts in C show the bi-orientation of the sister centromeres (arrowheads) at metaphase I. Inserts in D show the bi-orientation of the sister centromeres from univalents. The upper and lower inserts in E show the sister
chromatids separating from each other from a univalent and a bivalent, respectively. Interpretation models are illustrated at the last right column. The sister chromatids are indicated by identical greyscales, while dark and light grey indicate homologs. Putative crossovers are indicated by exchanged light and dark gray chromatin (asterisk). Dashed white and yellow lines indicate early sister chromatid cohesion loss and chiasmata resolution, respectively. Low quality of tubulin staining is due to the immuno-FISH method. Bar in E is equivalent to 5 µm.

During early meiosis II and at prophase II, in each cell a diploid number (2n=10) of individualized round-shaped chromatids is present. They display dispersed centromere signals (Fig. 6A). Then, when homologous non-sister chromatids associate in pairs towards metaphase II, the centromeric signals become visible as few cluster signals in the mid-region of each chromatid (Fig. 6B). Tubulin staining, especially during meiosis II, is challenging in Rhynchospora, thus, the distribution of spindle fibers is difficult to visualize. At metaphase II onset, the pairs of homologous non-sister chromatids show mostly a single cluster-holocentromere in the mid-region of each chromatid, which is stretched by spindle fibers from opposite poles (Fig. 6C, E-F inserts). The chromatids are of drop-like shape due to the tension caused by the spindle fibers (Fig. 6F). Surface rendering of metaphase II cells confirmed that the cluster-holocentromeres are mostly organized as a single cluster in the mid-region in each chromatid, occupying external and internal domains (Fig. 6I; Movie S9 and S10). During anaphase II the stretched homologous non-sister chromatids are then pulled to opposite poles (Fig. 6D, G). Finally, at telophase II, the tetrads contain four haploid nuclei with five chromatids each, showing five clustered centromeric signals (Fig. 6H). Thus, in contrast to the numerous cluster-holocentromeres observed in metaphase I, at metaphase II mostly a single cluster-holocentromere is present.
occupying a specific domain extending from the internal to external mid-region of each chromatid. Colchicine treatment did not disturb the patterns of cluster-holocentromere formation during MI and MII (Fig. S2E-F).

Due to the unusual arrangement of homologous non-sister chromatids at metaphase II, we asked whether the chromatid orientation is influenced by the telomeres. Since the 45S rDNA clusters are located terminally on three chromosome pairs of *R. pubera* (Sousa et al. 2011), we performed FISH with a 45S rDNA probe. The presence of the FISH signals always at the pole sides (*n = 27*) (Fig. 6J) supports the finding of Cabral et al. (2014), that preferentially the non-rDNA telomeres of the homologous non-sister chromatids associate. This indicates that the homologous non-sister chromatids are axially oriented during metaphase II, contrasting to the equatorial orientation of the bivalents at metaphase I.
Fig. 6 Cluster-holocentromere arrangement and homologous non-sister chromatid orientation during meiosis II. (A-B) Localization of the cenDNA (Tyba) during prophase II. (C-D) CENH3 and tubulin arrangement in (C) metaphase II and (D) anaphase II cells. (E-H) α-tubulin and cenDNA arrangement during (E-F) metaphase II, (G) anaphase II and (H) telophase II. (I) Surface rendering of metaphase II chromosomes showing the centromeres. (J) 45S rDNA localization on pairs of homologous non-sister chromatids. Low quality of tubulin staining is due to the immuno-FISH method. Bar in H is equivalent to 5 µm for A-H images.

To test whether a linear centromere structure becomes re-established after meiosis, the subsequent pollen mitosis was analyzed. In most plants all four male haploid products produce pollen. In contrast, in *R. pubera* a selective microspore abortion occurs, leading to pollen dispersal as pseudomonads (San Martin et al. 2013). Thus, at the end of meiosis three out of four haploid spores degenerate and a single one remains functional to develop the mature pollen. At late tetrad stage the four haploid nuclei decondense and the cluster-holocentromeres dissociate into smaller centromere units (Fig. 7A). Finally, a linear holocentromere organization appears at first pollen mitosis in all four cells of the pseudomonad, as identified after FISH with cenDNA (Fig. 7B). However, no groove-like structure is evident at this stage (Movie S11), perhaps due to differences in cell-type specific chromosome condensation. Remarkably, only the functional cell replicates, as indicated by double linear cenDNA signals. Instead, the degenerative cells possess only single chromatids (Fig. 7B). CENH3 linear signals were clearly present in the three degenerative nuclei, while the functional cells showed only weak, indistinct CENH3 signals (Fig. 7C). Whole-cell CENH3 fluorescence signal intensity measurements revealed that functional cells
have approximately half of the CENH3 content compared to degenerative cells (Table S3).

In summary, we conclude that the centromere unit arrangement differs between mitosis and meiosis in *R. pubera*. There is a transition from the mitotic linear organization within a groove to the cluster-holocentromeres arrangement at meiosis as summarized in Fig. 8. Finally, a linear holocentromere organization is re-established at first pollen mitosis, but without groove formation (Fig. 8B).

**Fig. 7** Re-establishment of a linear holocentromere structure in the *R. pubera* chromosomes during the pseudomonad development. Centromeres labelled by (A-B)
cenDNA (Tyba) and (C) CENH3. FC = functional cell, DC = degenerative cells. The arrowheads in B indicate both holocentromeres of a single replicated chromosome.

**Fig. 8** Model of differential holocentromere organization in the holocentric plant *R. pubera*. (A) Top and side (90° left turn) views of the centromere organization during mitosis and meiosis. During interphase, the centromere units are genome-wide dispersed in both somatic and meiotic cells. While the process of chromosome
condensation occurs, striking differences exist between mitotic and meiotic chromosomes. In mitotic chromosomes linear holocentromeres are formed within a groove, whereas both meiosis I and II chromosomes show a cluster-holocentromere organization and no grooves are visible. (B) Cell cycle dynamics of cluster-holocentromere organization and spindle fiber arrangement. During meiosis I, cluster-holocentromeres are oriented along the poleward surface of equatorially oriented bivalents, and the sister chromatids colocalize with spindle fibers from opposite poles (amphitelic attachment) causing their separation in anaphase I. During meiosis II the cluster-holocentromeres are localized in the mid-region of each chromatid. At this stage, pairs of homologous non-sisters chromatids are axially orientated and adopt a drop-like shape most likely due to the tension caused by the spindle forces at anaphase II. This causes the segregation of homologous chromatids. At telophase II each chromatid adopts a spheric shape with a strongly condensed cluster-holocentromere in the mid-region. During decondensation at late tetrads, the centromere units dissociate. Then, during first pollen mitosis they re-associate in such a way that a linear holocentromere is re-established. At this stage only the functional cell shows double centromere DNA signals caused by replication, whereas the CENH3 amount is clearly reduced compared to the degenerative cells.

Discussion
The mitotic holocentromere structures of *R. pubera* are not present during meiosis

Although *R. pubera* and *L. elegans* belong to sister families in the same order *Poales*, these holokinetic species show striking different meiotic centromere structures. While both species possess a linear holocentromere organization during mitotic metaphase, only *L. elegans* chromosomes exhibit the same structure also
during meiosis (HECKMANN et al. 2014). In contrast, R. pubera centromere units cluster during meiosis, but no distinct linear holocentromere within a groove is formed. A restoration of the linear holocentromere organization occurs after meiosis, during first pollen mitosis, although no groove is formed, in agreement with recent observations during pseudomonad development (ROCHA et al. 2016).

Why does the centromere organization differ between mitotic and meiotic chromosomes in R. pubera? The alternative association of centromeric units during meiosis may be due to a stronger degree of chromosome condensation and/or the absence of factors required for the linear arrangement of the holocentromeres. A deviating composition and dynamics of SMC proteins, such as cohesins and condensins could explain the striking divergences between mitosis and meiosis (ZAMARIOLA et al. 2014). Indeed, during R. pubera meiosis the chromatids lose their elongated shape, become round-shaped and do not form a groove. In contrast, similar chromatid and groove structures were found during mitosis and meiosis of L. elegans (HECKMANN et al. 2014). Poleward clustering of centromeres in R. pubera might help avoiding merotelic attachments to spindle microtubules. Clustering, however, is not likely a consequence of attached spindle microtubules pulling towards opposite poles, since a colchicine treatment of meiotic cells did not seem to disturb the formation of cluster-holocentromeres. In addition, a differential CENH3 loading dynamics during meiosis may act as adaptation to deal with holocentricity during meiosis. Indeed, the meiotic CENH3 loading may differ from mitosis in plants (RAVI et al. 2011; SCHUBERT et al. 2014). In contrast to mitosis, CENH3 deposition is biphasic during meiosis in rye and apparently linked with a quality check of CENH3 (SCHUBERT et al. 2014).
A different centromere structure during meiosis has been reported for a number of holocentric species. In *C. elegans*, the kinetochore activity involves a mechanism independent of CENH3 and CENP-C during meiosis I and II (Monen et al. 2005), and the chromosomes are ensheathed by microtubule bundles running laterally along their sides during female meiosis (Wignall and Villeneuve 2009; Schvarzstein et al. 2010). However, in male meiosis, the microtubule bundles are enriched at the bivalent ends facing polewards, indicating a telokinet-like activity (Wignall and Villeneuve 2009). The holocentric worm *P. univalens* restricts the kinetic activity of the microtubules to the heterochromatic terminal regions during male meiosis. These regions lack kinetochore structures and interact directly with the spindle fibers (Goday and Pimpinelli 1989; Pimpinelli and Goday 1989). Also in holocentric Heteroptera species, a restricted localized kinetic activity during meiosis I and II was reported (Perez et al. 2000; Papeschi et al. 2003). In most cases of telokinetic meiosis, a mechanism seems to be involved where both chromatid termini can acquire kinetic activity. This demonstrates a special case of kinetochore plasticity.

In the hemipteran genus *Oncopehus*, the presence of a holokinetic kinetochore plate during mitosis, but its absence during meiosis, was identified by electron microscopy. Additionally, multiple microtubule attachment sites were found at the meiotic chromosomes (Comings and Okada 1972). Similar findings were reported for other holocentric organisms, i.e. the nematode *Ascaris lumbricoides* (Goldstein 1977), the hemiptera *Rhodnius prolixus* (Buck 1967) and *Graphosoma italicum* (Rufas and Gimenez-Martin 1986) and the Lepidoptera *Bombyx mori* (Friedlander and Wahrman 1970). In contrast, in the holocentric scorpion *Tityus bahiensis*, a kinetochore plate throughout meiosis was found, while in the spiders *Dysdera crocata* and *Segestria florentina* kinetochore plates were evident only during meiosis.
Thus, the absence of a kinetochore plate during meiosis seems to occur rather frequently among holocentric organisms and was postulated to be related to the restriction of kinetic activity and terminalization of chiasmata necessary for a normal progression of meiosis in those organisms (Comings and Okada 1972; Pimpinelli and Goday 1989). In addition, it is interesting to notice that all holocentric insects lacking kinetochore plates during meiosis also lack CENH3 and CENP-C genes, and occasionally some other inner kinetochore proteins, whereas most of the outer kinetochore genes were still present (Drinnenberg et al. 2014). Whether the lack of CENH3 and CENP-C causes a mis-assembly of kinetochore plates during meiosis in these organisms is still unknown.

Thus, the meiotic holocentromeres in R. pubera are unique as it is the only holocentric species so far showing a differential centromere organization in mitosis and meiosis, while spindle fibers attach to its centromere units composed by CENH3 and CENP-C. As discussed above, most organisms showing differential centromere organization either lack CENH3 and CENP-C (Drinnenberg et al. 2014) or these proteins do not play a role in chromosome segregation during meiosis (i.e. C. elegans). In contrast, a similar organization of mitotic and meiotic holocentromeres was found in L. elegans, although no CENP-C antibody has been generated and tested for this species (Heckmann et al. 2014).

A linear holocentromere organization is not required for the reversion of the chromatid segregation events during meiosis in holokinetic species

We confirmed the previously reported unusual process of meiosis in R. pubera (Cabral et al. 2014) by showing a bipolar sister centromeres orientation and their attachment to microtubules from opposite spindle poles in meiosis I (amphitetic...
attachment), the segregation of the sister chromatids to opposite poles already
during anaphase I, and the alignment and segregation of homologous non-sister
chromatids only during the second meiotic division. Remarkably, a differential
orientation of cluster-holocentromeres was observed from meiosis I and II. While
during meiosis I the cluster-holocentromeres were observed mostly accumulated
along the poleward surface of the bivalents, in meiosis II the cluster-holocentromeres
were mostly visible as a single cluster in the mid-region of each chromatid. Notably,
the homologous non-sister chromatids are preferentially associated by their non-
rDNA termini at metaphase II as already described by CABRAL et al. (2014). The
results indicate a distinct orientation and interaction of spindle fibers with the cluster-
holocentromeres between meiosis I and II. While during metaphase I the bivalents
orient perpendicular to the spindle poles, during metaphase II the pairs of
homologous non-sister chromatids orient with their longer axis in parallel to the
spindle poles.

Moreover, our results show that a linear holocentromere organization as found in L.
elegans is not required for the reversion of the segregation events of the
sister/homologous chromatids during inverted meiosis. Actually, considering an end-
to-end interaction of the homologous non-sister chromatids in metaphase II, the
linear structure is compatible with proper chromatid segregation towards opposite
poles because Luzula chromosomes maintain a U-shape conformation in meiosis II.
In fact, the highly clustered holocentromere found at metaphase II and anaphase II in
R. pubera seems to present an alternative solution to reduce the risk of merotelic
attachment of microtubules. However, while no missegregation was found during
meiosis I in R. pubera, it was reported that 19.5% of all meiosis II products had
incorrect chromosome numbers (CABRAL et al. 2014). In the nematode C. elegans,
the chromokinesin KLP-19 counteracts persistent merotelic attachments (Powers et al. 2004). Whether in *R. pubera* a similar correction mechanism exists is unknown. Although merotelic attachments might cause missegregation during meiosis II of *R. pubera*, Cabral et al. (2014) suggested that pairs of homologous non-sister chromatids may have failed to connect to each other, thus leading to missegregation in meiosis II.

During first pollen mitosis, CENH3 signals were much stronger in the degenerative cells, while the functional cell showed a weak and indistinct labeling. These differences might be explained by the absence of *de novo* incorporation of CENH3 molecules after the exit from meiosis. Thus, preexisting CENH3 could be partitioned equally between duplicated sister centromeres as a result of cell replication, which occurs only in the functional cell (evidenced by double lines of cenDNA signals). Thereby, a fixed number of CENH3 molecules split between the generative and vegetative nucleus, which explains the 50% of CENH3 signal intensity found in functional cells compared to degenerative cells. Alternatively, active CENH3 removal in the functional haploid cell after meiosis exit could cause the reduction of CENH3 molecules as found in rye (Schubert et al. 2014). The latter is possible, since removal of CENH3 has been observed in differentiated and in vegetative pollen cells of *A. thaliana* (Schoft et al. 2009; Merai et al. 2014). Furthermore, the weak CENH3 signals observed in the functional cell suggests that a reduced amount of CENH3 is still sufficient for proper chromosome segregation (Liu et al. 2006; Lermontova et al. 2011; Karimi-Ashtiyani et al. 2015).

What does the unusual meiotic centromere arrangement of *R. pubera* implicate?
The inappropriate occurrence of crossovers in the proximity of the primary constriction of monocentric chromosomes effects negatively the meiotic chromosome segregation by influencing the centromeric cohesion (Talbert and Henikoff 2010; Vincenten et al. 2015). Accordingly, the occurrence of very few crossovers is reported for holocentric organisms, generally one or two per rod- and ring-bivalent, respectively, mostly located at the non-centromeric terminal regions (Cuacos et al. 2015). This is also true for R. pubera, in which chiasmata occur terminally. In this case, recombined bivalents are resolved because of the loss of sister chromatid cohesion in anaphase I. Furthermore, R. pubera faces another challenge during meiosis, since its unusual centromere arrangement of meiotic chromosomes could cause a high risk of misorientation during meiosis I. However, no chromosome fragmentation or anaphase bridges were observed during the meiosis of R. pubera. But, the unusual centromere organization might be associated with decrease in recombination, what could be the cause of the frequent (3.5%) occurrence of univalents in R. pubera. In fact, this could also explain the occurrence of univalents in the achiasmatic meiosis of R. tenuis (Cabrál et al. 2014). Thus, it seems that the meiosis of Rhynchospora is adapted to solve potential meiotic errors due to the unusual centromere arrangement.

The Cf19 complex of yeast (also known as the Constitutive Centromere-Associated Network CCAN in other organisms) prevents meiotic double strand breaks (DSBs) proximal to the centromeres, which are essential to initiate recombination (Vincenten et al. 2015). Nevertheless, although meiotic DSBs are suppressed at core centromeric regions in yeast, they frequently occur only a few kilobases away from the centromeres (Buhler et al. 2007; Pan et al. 2011). In Rhynchospora, meiotic DSBs are normally formed and processed in early prophase I, as evidenced by the
presence of multiple RAD51 foci (CABRAL et al. 2014). Other meiotic events typical of
the first meiotic prophase, such as the meiotic axis formation, appears normal in R. pubera, since the axial element protein ASY1 showed the typical pattern known from monocentric species (CABRAL et al. 2014). Therefore, it is interesting that the meiotic cluster-centromere arrangement of R. pubera does not disturb DSB formation, axis architecture nor synaptonemal complex formation. Thus, to deal with its centromere architecture during meiosis a very accurate regulation of meiotic recombination is likely to exist in R. pubera.

In conclusion, the holocentromeres of R. pubera are unique in respect of their differential organization during mitosis and meiosis. Our results reinforce the idea of high centromere plasticity among holocentric organisms and offer a novel model for understanding centromere evolution and function among eukaryotes.

Data availability

Antibodies are available upon request. Sequence data are available at GenBank and the accession numbers are listed in the Materials and Methods session and in Table S2.

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Competing interests

The authors declare no conflict of interests.

Deposition data

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References


