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RESEARCH ARTICLE

Holokinetic centromeres and efficient telomere healing enable rapid karyotype evolution

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Abstract Species with holocentric chromosomes are often characterized by a rapid karyotype evolution. In contrast to species with monocentric chromosomes where acentric fragments are lost during cell division, breakage of holocentric chromosomes creates fragments with normal centromere activity. To decipher the mechanism that allows holocentric species an accelerated karyotype evolution via chromosome breakage, we analyzed the chromosome complements of irradiated *Luzula elegans* plants. The resulting chromosomal fragments and rearranged chromosomes revealed holocentromere-typical CENH3 and histone H2AThr120ph signals as well as the same mitotic mobility like unfragmented chromosomes. Newly synthesized telomeres at break points become detectable 3 weeks after irradiation. The presence of active telomerase suggests a telomerase-based mechanism of chromosome healing. A successful transmission of holocentric chromosome fragments across different generations was found for most offspring of irradiated plants. Hence, a combination of holokinetic centromere activity and the fast formation of new telomeres at break points enables holocentric species a rapid karyotype evolution involving chromosome fissions and rearrangements.

Keywords Chromosome fusion and fission · De novo telomere synthesis · Holocentric chromosome · Holocentric genome evolution · *Luzula elegans*

Introduction

Fragmentation of monocentric chromosomes by ionizing irradiations (e.g., γ-, X-rays) and UV irradiation causes the formation of centric and acentric fragments. Acentric fragments due to the absence of kinetochores do not segregate and get consequently lost during mitosis which might lead to lethal mutations. In contrast, breakage of holocentric chromosomes generates mainly fragments possessing an active centromere, and thus, fragments can segregate normally in somatic cells (Hughes-Schrader and Ris 1941). Holocentric chromosomes lack a primary constriction and form holokinetic centromeres distributed along almost the entire length of the chromatids. In addition, in holocentric species, irradiation-induced chromosome rearrangements such as reciprocal translocations do not result in dicentric chromosomes, which often fail to segregate properly if both centromeres are active (McClintock 1939). Therefore, irradiation of holocentric species rarely results in anaphase bridge and micronuclei formation (Hughes-Schrader and Ris 1941; Nordenskiöld 1964; Pazy and Plitmann 1994). Consequently, chromosome fragment behavior was analyzed to demonstrate the presence of holokinetic centromeres in different species of green algae (Godward 1954), flowering plants (Håkansson 1954), nematodes (Albertson and

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Thomson 1982), and arthropods (Tempelaar 1979). The sporadic and polyphyletic occurrence of holocentricity in the tree of life suggests that holocentric centromere evolved from monocentric chromosomes at least 13–times independently in different clades (Melters et al. 2012).

Fragments of holocentric chromosomes are inherited stably throughout many generations and can give rise to a stable progeny (Nordenskiöld 1962, 1963, 1964; LaChance and Degrugillier 1969). In the genus *Luzula*, the haploid chromosome number varies in a broad range, and species with 3, 6–16, 18, 21, 23, 24, 26, 31, 33, 35, 36, and 42 chromosomes were reported (Nordenskiöld 1951; Kuta et al. 2004; Záveská Drábková 2013). A comparable situation was found for *Cyperus* plant species or holocentric butterflies of *Nymphalidae* with nearly continuous chromosome numbers from 5 to 134 (reviewed in Bureš et al. 2013). Moreover, within one holocentric species, the number of chromosomes can vary between different individuals as shown for plant species *Carex blepharicarpa* with 2n=26–32 and 41 (Hoshino and Okamura 1994), *Luzula multiflora* with 2n=12, 18, 24, 28, 36, and 48 (Bolkhovskikh et al. 1969), or *Eleocharis kamtschatica* with 2n=41–47 (Yano and Hoshino 2006). It has been speculated that stably inherited chromosome fragments and the lack of dicentric translocation products are the reasons that number and size of chromosomes may vary considerably and may be tolerated in species with holocentric chromosomes.

Obviously, due to the fragmentation of holocentric chromosomes in different species of the genus *Luzula*, a negative correlation between chromosome number and chromosome size was found. When the chromosome number is doubled from 12 to 24, the length of chromosomes is about halved (Nordenskiöld 1951). Similarly, in *Juncus biglumis,* two cytotypes with 2n=60 and 2n=120 chromosomes were observed. Chromosomes of the 2n=60 cytotype are about twice as big as the chromosomes of the cytotype with 2n=120 while the relative DNA content differs by only 6 % between them (Schönswetter et al. 2007). Interestingly, in *Luzula* hybrids derived from parents possessing small and large chromosomes, meiotic pairing occurs between one large and two half-sized chromosomes (Nordenskiöld 1961).

Malheiros-Garde and Garde (1950) and Nordenskiöld (1951) postulated the occurrence of so-called chromosome "fusion and fission" events during the evolution as an explanation for differently sized chromosomes in holocentric species. However, the term "chromosome fusion" should be used with caution because fusions *sensu stricto* imply the combination of two chromosomes without any loss of chromatin, which is usually prevented by telomeres (Schubert and Lysak 2011). Interstitial telomere repeats as indication of translocations with a break point inside with telomere repeat arrays were found in the spike rush *Eleocharis subarticulata* (Da Silva et al. 2005). In contrast, other holocentric species, e.g., aphids (Monti et al. 2011), the plants *Luzula luzuloides* (Fuchs et al. 1995), and *Rhynchospora tenuis* (Vanzela et al. 2003), the and cabbage moths *Mamestra brassicae* (Mandrioli 2002), did not display interstitial telomeres. Likely, the so-called fusion events are based on translocations and subsequent loss of small translocation products including telomeres resulting in terminally truncated "fused" chromosomes. In addition to chromosome fragmentation and translocation, polyploidy and proliferation/removal of high-copy sequences are mechanisms involved in the genome evolution of holocentric species (Kuta et al. 2004; Bačič et al. 2007; Bozek et al. 2012; Zedek et al. 2010; Záveská Drábková 2013).

Beside the segregation failure due to centromere loss, broken ends of centric fragments of monocentric chromosomes may be mis-repaired, yielding dicentric and ring chromosomes causing breakage–fusion-bridge (BFB) cycles (; Muller 1938; McClintock 1941, 1942). Alternatively, chromosome fragments can be healed by adding telomeric sequences to the broken chromosome ends (McClintock 1941; Day et al. 1993). Telomeres are synthesized by a specialized reverse transcriptase, the telomerase, which can replenish already existing telomeres or add new telomeric sequences directly to non-telomeric DNA, for example, at the break points of chromosome fragments (reviewed in Melek and Shippen 1996). Telomeres are added gradually, and the start of the process might require passing through a certain number of cell cycles and/or a certain developmental stage (Tsujimoto 1993; Britt-Compton et al. 2009).

Alternatively, broken chromosome ends can be healed by a telomerase-independent mechanism termed telomere capture. In this case, broken chromosomes are stabilized by a transfer of telomeres from unbroken chromosomes to the broken ends likely via a conversion-like recombination process (Meltzer et al. 1993), which may also occur between broken and intact homologues (Slijepcevic and Bryant 1998; Lundblad 2002).

Here, we investigated the consequences of fragmentation of holokinetic chromosomes across three generations. Furthermore, we address the question whether or not, and if so, when telomere healing of chromosome fragments occurs. For this purpose, we selected the wood rush *Luzula elegans* Lowe (formerly *L. purpurea* Link), a plant species for which fragmentation caused by X-irradiation has been reported previously (Nordenskiöld 1962, 1963, 1964).

L. elegans (*Juncaceae*) is a self-fertilizing holocentric species with a diploid chromosome number of 2n=6 (3.81 Gbp/1C) (Heckmann et al. 2013). The chromosomes of this species are characterized by a longitudinal CENH3-positive groove-like structure along each sister chromatid (throughout mitosis and meiosis), flanked by *Arabidopsis*-type telomeres (Heckmann et al. 2011, 2014; Wanner et al. 2015). As an adaption to the holokinetic centromere organization,

L. elegans performs an inverted sequence of meiotic sister chromatid segregation events (Nordenskiöld 1962; Heckmann et al. 2014).

Materials and methods

Plant material and X-ray irradiation

Seeds of *Luzula elegans* Lowe (2n=6) (herbarium vouchers of IPK Gatersleben: GAT 7852–7856) were germinated on wet filter paper at 21 °C. Three leaf stage plantlets (28 days old) were irradiated with various doses ranging from 10 to 30 Gy with an X-ray apparatus (Yxlon, International Hamburg). The dose rate amounted to 0.9 Gy/min. Subsequently, plantlets were harvested in 7-day intervals and fixed for 45 min in ice-cold 4 % (*w*/*v*) paraformaldehyde in 1× MTSB buffer (50 mM PIPES, 5 mM $MgSO_4$, and 5 mM EGTA, pH 7.2) for immunolabeling or in ethanol–acetic acid (3:1, *v*/*v*) fixative for fluorescence in situ hybridization (FISH). To induce flowering, the plants were transmitted to soil and subjected to vernalization for a minimum of 3 months (10 h light/14 h dark, 4 °C). Afterwards, the plants were grown under long-day conditions (13 h light/11 h dark, 20 °C/16 °C). Flower buds were collected and fixed as described above. The M1 offspring was a selfing product of irradiated plants.

Fluorescence in situ hybridization

Mitotic chromosome spreads, derived from acetocarmine-stained root and apical meristems, were prepared from fixed plantlets by squashing (Houben et al. 1999). Meiotic chromosomes were prepared from fixed flower buds (Heckmann et al. 2014). PCR generated FISH probes for the subtelomeric satellite repeat LeSAT7, and telomeres were labeled with ChromaTide Texas Red-12-dUTP or Alexa Fluor 488-5-dUTP (http://www.invitrogen.com) by nick translation. FISH was performed according to (Heckmann et al. 2013).

Indirect immunolabeling

Fixed flower buds were used to prepare spreads by squashing. Immunostaining was performed as described (Houben et al. 2007). The following dilutions of primary antibodies were used: 1:100 of rabbit anti-LnCENH3 (Nagaki et al. 2005) and 1:200 of rabbit anti-H2AThr120phos (Abcam, www.abcam.com). A FITC-conjugated anti-rabbit Alexa488 antibody (Molecular Probes, http://www.invitrogen.com) at 1:400 dilution was used as secondary antibody.

Microscopy

Fluorescence images were captured using an Olympus BX61 microscope equipped with an ORCA-ER CCD camera (Hamamatsu). Deconvolution of image stacks of 10 slices each and maximum intensity projections were done using the program AnalySIS (Soft Imaging System). All images were acquired in grey scale and afterwards pseudo-colored and merged with Adobe Photoshop CS5 (Adobe). To achieve an optical resolution of ~120 nm (super-resolution), we applied structured illumination microscopy (SIM) using a C-Apo 63×/1.2W Korr objective of an Elyra PS.1 microscope system and the software ZEN (Carl Zeiss GmbH). A SIM image stack was used to produce the 3D movie by the Imaris 8.0 (Bitplane) software.

Flow cytometric genome size measurement

The *Luzula* DNA content was estimated using young, fresh leaves according to (Fuchs et al. 2008) using *Pisum sativum* 'Viktoria, Kifejtö Borsó' (Genebank Gatersleben accession number PIS 630; 2C=9.09 pg) (Doležel et al. 1998), as an internal reference standard. Measurements were performed either on a FACStar^{PLUS} flow sorter (BD Biosciences) equipped with an argon ion laser INNOVA 90C (Coherent) adjusted to 514 nm or on a CyFlow space flow cytometer (Partec) equipped with a 532 nm solid-state laser. Each measurement was repeated at least two times on different days.

Analysis of telomerase activity according to the telomere repeat amplification protocol

Three leaf stage plantlets and flower buds of *L. elegans*, and 7-day seedlings of *Arabidopsis thaliana* were manually homogenized in extraction buffer (Fitzgerald et al. 1996; Sykorova et al. 2003). Crude extracts obtained after centrifugation were 5× and 10× diluted for analysis of telomerase activity as described (Fitzgerald et al. 1996; Fajkus et al. 1998). Briefly, 1 μl of 10 μM substrate primer TS21 (5′GACAATCCGT CGAGCAGAGTT3′) was mixed with 1 μl of diluted crude protein extract, and elongation of the primer by the telomerase proceeded for 45 min at 26 °C in 25 μl reaction buffer (Fitzgerald et al. 1996). Telomerase was heat inactivated (5 min, 94 °C); 1 μl of 10 μM reverse primer TelPr (5′ CCGAATTCAACCCTAAACCCTAAACCCTAAACCC3′) and 2U of DyNAzymeII DNA polymerase (Finnzymes) were added, and extension products were amplified in PCR (35 cycles of 95 °C/30 s, 65 °C/30 s, 72 °C/30 s; final extension 72 °C/5 min). Aliquot samples of telomere repeat amplification protocol (TRAP) reactions were analyzed a on 12.5 % polyacrylamide gel in 0.5×TBE buffer. Gels were stained by GelStar nucleic acid gel stain (LONZA) and signals were visualized using the LAS-3000 system (FujiFilm).

Results

X-ray radiation induces aberrations in holocentric chromosomes in a dosage-dependent manner

Three leaf stage plantlets of *L. elegans* were X-ray irradiated with 10, 20, 25, and 30 Gy to induce fragmentation of the holocentric chromosomes. In agreement with previous findings (Prakken 1959; Li et al. 2010), DNA double-strand breaks occurred more frequently with increasing radiation dosage. Both, the non-irradiated plant and the plant irradiated with 10 Gy did not show any chromosome fragmentation and possessed three equally sized chromosome pairs. FISH with the *Arabidopsis*-type telomere $(TTTAGGG)_n$ repeat and the satellite repeat LeSAT7, which clusters at the ends of all *L. elegans* chromosomes (Fig. 1a) (Heckmann et al. 2011), confirmed the absence of major structural changes in these low- or non-radiated samples. In contrast, irradiation doses of 20, 25, and 30 Gy induced numerous chromosome fragments detectable in metaphase cells 1 day after irradiation. Whereas 7 chromosomes/fragments was the most frequent number in plants irradiated with 20 Gy (66 %, n=61) and 25 Gy (70 %, n=50), an increase to 9 was found in the plant irradiated with 30 Gy (54 %, n=67) (Fig. 1b). Irradiated plants were cytogenetically heteromorphic exhibiting a variability in the newly formed chromosome/fragment sizes between cells and plants. In the plant irradiated with 25 Gy, we observed in addition to small fragments also an abnormally large chromosome (Fig. 1c). The absence of interstitial telomeres or LeSAT7 signals indicates that this chromosome might be the result of a translocation event combining two or more fragments.

Chromosome fragments containing a holokinetic centromere are stabilized by de novo formed telomeres

In order to test whether the fragments and the rearranged chromosomes possess normal centromere activity, the position of kinetochores was evaluated by immunostaining with CENH3- or histone H2AThr120ph-specific antibodies. Both antibodies label only functionally active centromeres (Allshire and Karpen 2008; Kawashima et al. 2010). Colocalization of the centromeres and the *Arabidopsis*-type telomeres revealed telomere signals at both ends of the longitudinal centromeres (Fig. 2a, Supporting Information Movie S1). Hence, the centromere spans over the entire chromosome, from telomere to telomere in non-irradiated plants. The CENH3-negative regions appearing distal to the telomere signals likely represent out-looped subtelomeric chromatin.

The chromosome fragments showed similar CENH3 and H2AThr120ph signals as unfragmented chromosomes (Fig. 2b, c). Extraordinary long chromosomes, presumably products of translocation events, revealed centromere proteins along the entire chromosome length (Fig. 2c). No interstitial signal gap was found by the centromere labeling. These observations and the absence of micronuclei indicate that fragmented and translocated chromosomes segregate normally in somatic cells due to the presence and activity of the holokinetic centromeres.

To study whether the ends of broken chromosomes are stabilized by de novo formed telomeres, in situ hybridization with the *Arabidopsis*-type telomere probe was performed in plants irradiated with 20 Gy 7, 14, and 21 days after irradiation. To distinguish between pre-existing and newly formed telomere sites, the subtelomere-specific probe LeSAT7 was

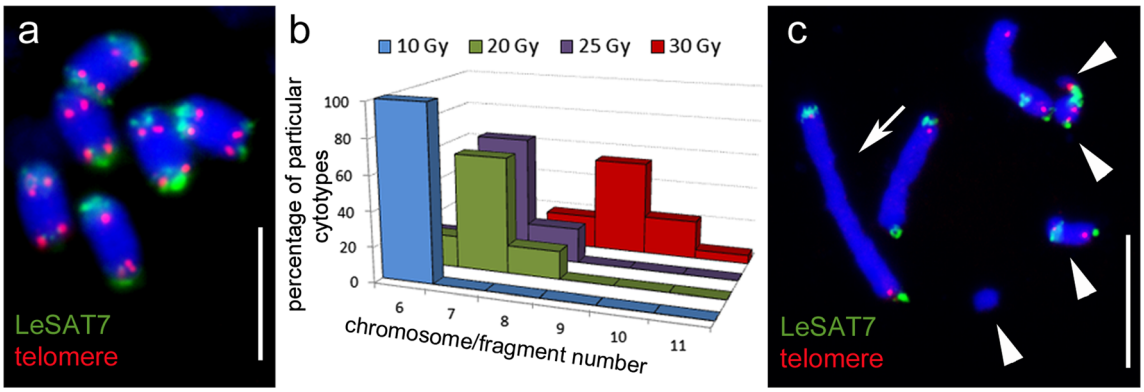

Fig. 1 X-ray irradiation induces chromosome fragmentation in *L. elegans*. **a** Non-irradiated mitotic metaphase with three equally sized chromosome pairs after FISH with the *Arabidopsis*-type telomere and the terminal satellite repeat LeSAT7. **b** Chromosome fragmentation rises with increasing radiation dosage. The plant irradiated with 10 Gy did not show any fragmentation and possessed six chromosomes. The most frequent number of chromosomes/fragments in the plant irradiated with 20 Gy was 7 (40 cells). In 11 cells 6 and in 10 cells 8 chromosomes could be counted. The most frequent chromosome number in the plant irradiated with 25 Gy was 7 (35 cells), followed by 8 (10 cells) and 6 (5 cells). In the plant irradiated with 30 Gy, cells with 9 (36 cells), 10 (15 cells), 8 (13 cells), and 11 (3 cells) chromosomes/fragments were observed. **c** Mitotic metaphase of a plant irradiated with 25 Gy possessing seven chromosomes/fragments of different size after FISH with the *Arabidopsis*-type telomere probe and the terminal satellite repeat LeSAT7. *Arrowheads* indicate the chromosome fragments. The *arrow* points to an abnormally large chromosome, presumably the product of a translocation event. DNA was counterstained with DAPI (*blue*). *Bars*= 10 μm

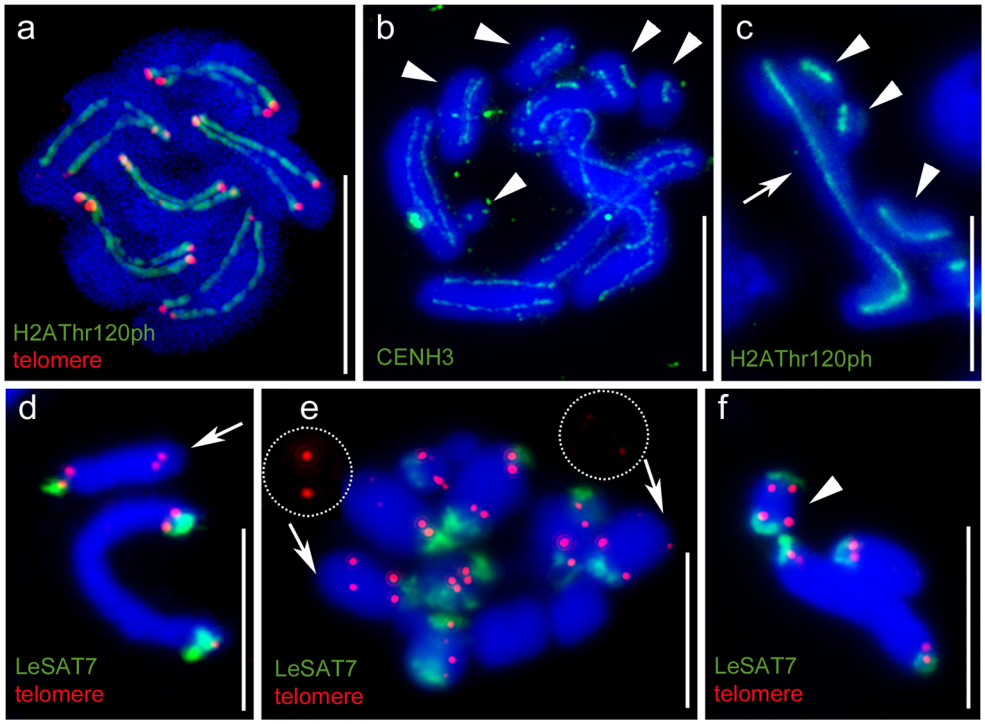

Fig. 2 Chromosome fragments of *L. elegans* possess holokinetic centromeres and de novo formed telomeres. **a** SIM of double labeled non-irradiated mitotic metaphase chromosomes with anti-H2AThr120ph immunostaining and FISH using the *Arabidopsis*-type telomere probe. The centromeres span the entire chromosome length from telomere to telomere (see also Supporting Information Movie S1). Immunolabeling of irradiated cells with anti-CENH3 (**b**) and anti-H2AThr120ph (**c**). Fragmented (*arrowheads*) and translocated (*arrow*) chromosomes possess active holocentromeres. **d** FISH with the terminal satellite LeSAT7 and telomere repeats (*red*) enable to distinguish between pre-existing telomeres (carrying signals of both probes) and the newly synthetized telomeres (carrying only telomere signals, *arrow*). **e** The signal intensities of the newly synthesized telomeres differ between the fragmented chromosome ends (see further enlarged telomere signals, arrowed). **f** A chromosome fragment with pre-existing telomeres at both termini (*arrowhead*). Chromatin was counterstained with DAPI (*blue*). *Bars*=10 μm

used in addition. Telomeric regions carrying both LeSAT7 and telomere repeats were assumed to be the pre-existing chromosome ends, while ends lacking LeSAT7 but exhibiting telomere signals were considered as those with newly synthesized telomeres (Fig. 2d). The first chromosome fragments exhibiting a newly formed telomere were found 21 days after irradiation. At this time point, newly formed telomeres were detectable at 51 % of 37 analyzed fragments. The hybridization signal intensity differed severely among the newly formed telomeres (Fig. 2e). Around 3 months after radiation, when the plants enter meiosis, the intensity of telomere signals was compared between chromosome fragments carrying a pre-existing and a newly formed telomere. In 70 % of 69 analyzed fragments, the hybridization intensity of newly formed telomeres was weaker than that of the pre-existing ones. In 17 % of the fragments, no obvious difference between "new" and "old" telomere sites was detectable, and 9 % of the newly formed telomeres showed an even more intense hybridization signal than the "pre-existing" ones. Only 4 % of chromosome fragments revealed no telomere signal at the newly formed chromosome ends. In addition, small chromosome fragments carrying telomeres and LeSAT7 repeats at both ends could be observed (Fig. 2f), likely due to the fusion of two subtelomeric regions.

To test whether the newly formed telomeres may be the product of active telomerase, we used a PCR-based TRAP assay which permits the detection of telomerase activity in vitro. Semi-quantitative analysis of TRAP products showed detectable levels of telomerase in extracts isolated from non-irradiated *L. elegans* seedlings and flower buds. Regular ladders of TRAP product showing the same periodicity as in the *A. thaliana* sample which was used as a positive control were reproducibly detected in both *L. elegans* tissues (Fig. 3). This observation suggests that de novo synthesized telomeres observed in somatic and generative cells are the likely result of telomerase-mediated healing process.

Successful transmission of holocentric fragments across several generations

L. elegans performs an inverted meiosis meaning that sister chromatids separate from each other during anaphase I and homologous non-sister chromatids separate during anaphase II (Heckmann et al. 2014). For several *Luzula* species, the

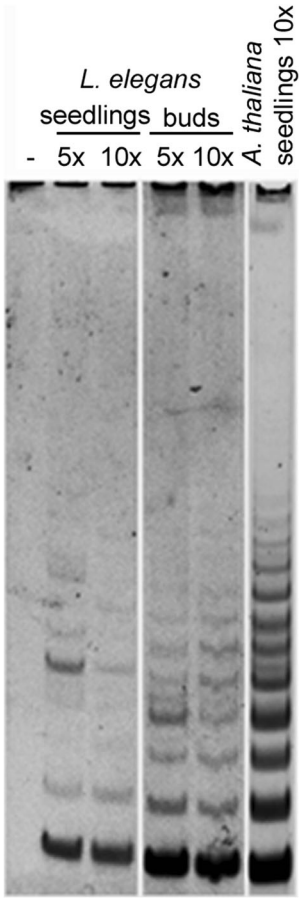

Fig. 3 Telomerase activity in seedlings and flower buds assayed by TRAP. Seedlings and flower buds of *L. elegans* show telomerase activity, as evidenced by the 7-bp incremental TRAP ladder. Total protein extracts from seedlings and flower buds were analyzed in 5× and 10× dilutions. An *A. thaliana* extract was used as a positive control. − negative control (no extract in the reaction)

stable transmission of holocentric chromosome fragments was described (Nordenskiöld 1962, 1963; Kusanagi 1973). Irradiated plants exhibited a bouquet formation in zygotene, like non-irradiated ones (Fig. 4a). However, at prometaphase I, multivalent configurations interconnected by terminal satellite repeats were detected, which have never been observed in non-irradiated plants (Fig. 4b). Additionally, we found the terminal satellite at an interstitial position as a product of a translocation event (Fig. 4c).

To further investigate whether meiosis is disturbed by the irradiation-induced aberrations, we determined the DNA content of the progeny of the plant irradiated with 20 Gy. Nineteen out of 20 analyzed M1 plants revealed a genome size comparable to non-irradiated plants (3.81 Gbp/1C± 1.5 %) indicating a balanced segregation of all chromosomes including fragments. All chromosome fragments in the self-progeny of the irradiated plant possess telomeric repeats at their newly formed chromosome ends (Fig. 4d). In one plant, a 7 % larger genome was detected (4.08 Gbp/1C). Analyses of this plant in prometaphase I showed three chromosome fragments of different size (Fig. 4e) in addition to three bivalents. The corresponding tetrads revealed unequal numbers of telomere and LeSAT7 signals in daughter cells indicating a random segregation of these chromosome fragments. Furthermore, in 20 % of 100 examined tetrads of this M1 plant, micronuclei were observed (Fig. 4f) but not present in the other M1 plants. The DNA content of the M2 progeny of the M1 plant with 7 % bigger genome size ranged from 7.74 pg/2C (−0.76 %) to 8.87 pg/2C (+14 %) compared to 7.80 pg/2C of non-irradiated plants. In addition, the number and size of chromosomes and fragments in the M2 plants varied, but all chromosome and fragment ends contained telomere repeats. Plants possessing 3.35, 6.46, and 11.03 % bigger genomes harbored chromosome complements with 2n=6+2 small fragments, 2n=6+2 big+2 small fragments, and 2n=7+1 small fragment, respectively (Fig. 4g, h, i) indicating a stable transmission of fragments independent of their size. Despite this severe variation in DNA content and karyotype constitution, no obvious phenotypic differences were observed.

The balanced segregation of the chromosome fragments to the progeny in 95 % of the cells indicates a correct process of inverted meiosis independent of irradiation induced fragmentations, translocations, and multivalent formation. The presence of telomere repeats at all broken ends in the progeny implies a significant role of telomere de novo formation for stable fragment transmission across generations.

Discussion

Species with holocentric chromosomes are characterized by a rapid karyotype evolution (Bureš et al. 2013). In contrast to organisms with monocentric chromosomes where acentric fragments are mostly lost during cell division, the breakage of holocentric chromosomes creates fragments with normal centromere activity. Therefore, no lagging anaphase chromosomes and micronuclei occur, and chromosome breakage and translocation events play an important role in the fast karyotype evolution of holocentric species (Heilborn 1924; Brown et al. 2004; Kuta et al. 2004; Da Silva et al. 2008; Hipp et al. 2009).

Using centromere-specific markers, we demonstrate that chromosome fragments independent of their size possess centromere activity along their sister chromatids. No abnormalities were found during mitotic divisions, similar as in γ-irradiated holocentric plant species *Rhynchospora pubera* (Vanzela and Colaço 2002) and the holocentric nematode *Caenorhabditis elegans,* where only very small fragments were rarely lost (Albertson and Thomson 1982). However, a holokinetic centromere is likely not sufficient to stabilize broken chromosomes as telomere-free chromosome ends are prone to “fuse”, and thus, form ring chromosomes when

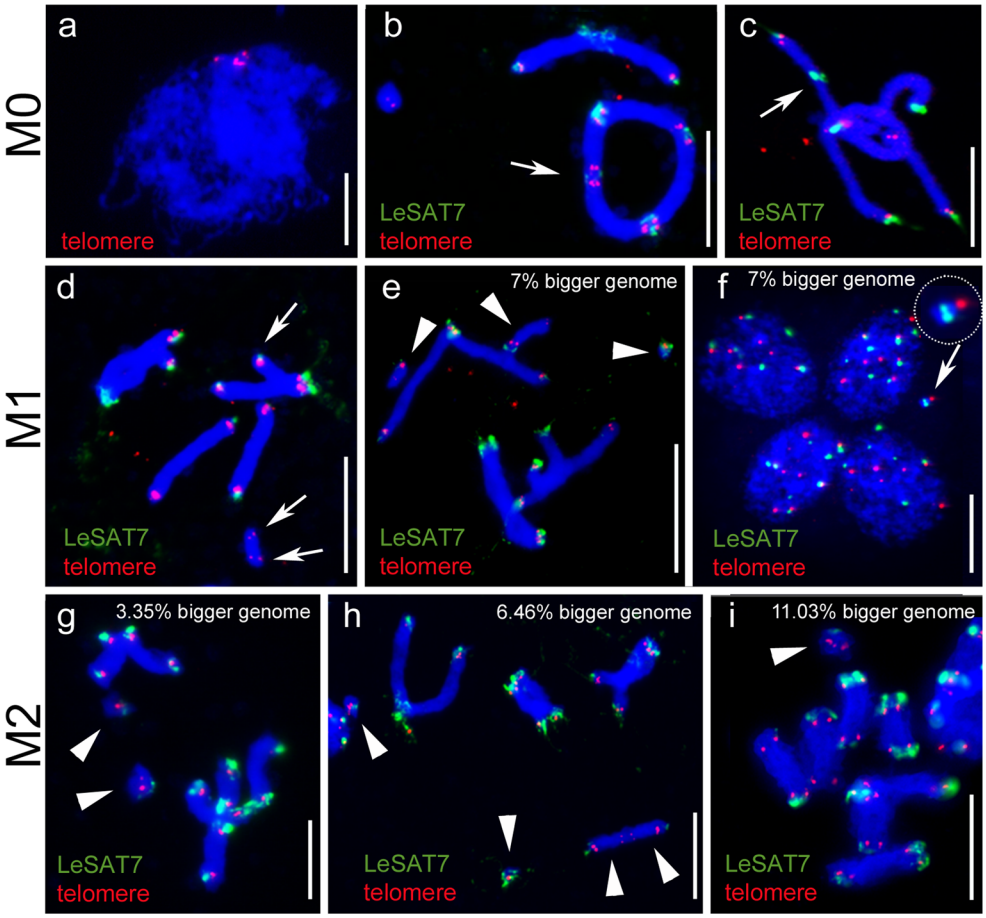

Fig. 4 Holocentric fragments in mutants (M0–M2) are stably transmitted to the next generation. **a** The formation of a bouquet-like configuration during zygotene of M0 mutants is not impaired by irradiation. **b** A multivalent configuration (*arrow*) is present at prometaphase I of irradiated M0 plants. **c** A translocation event caused by irradiation is indicated by the interstitial localization of the terminal satellite LeSAT7 (*arrow*). **d** Somatic metaphase of a mutant offspring (M1) with stably transmitted chromosome fragments (2n=7) and a similar genome size as non-irradiated plants. All chromosome fragments possess telomeric repeats at their newly formed chromosome ends (*arrows*). **e** Prometaphase I of a mutant offspring with a 7 % larger genome possessing three bivalents and three fragments of different sizes (*arrowheads*). **f** Tetrads of the same mutant showing a micronucleus (further enlarged and arrowed). **g**, **h**, **i** M2 individuals of the progeny of the mutant with the 7 % larger genome revealed different combinations of the fragments (*arrowheads*) occurring in the mother plant. Number and size of fragments correlate with the estimated genome size: 2n=6+2 small fragments (3.4 % enlarged genome), 2n=6+2 big+2 small fragments (6.5 % enlarged genome), and 2n=7+1 small fragment (11.0 % enlarged genome). FISH with the terminal satellite LeSAT7 and the telomere repeat. Chromatin was counterstained with DAPI (*blue*). *Bars*=10 μm

fragments without terminal telomeric repeats are involved (McClintock 1941, 1942). We did not observe ring chromosomes, probably due to rapid telomere healing. Ring chromosome formation was also not present in irradiated *C. elegans* cell (Albertson and Thomson 1982). However, in the holocentric common spikerush, *Eleocharis palustris,* ring chromosomes were found after irradiation (Håkansson 1958).

In our study, cytologically detectable small telomere repeats at ~50 % of broken termini appeared already 21 days after irradiation. This indicates a telomerase-based mechanism of chromosome healing in *Luzula*, similar as described for monocentric human (Chabchoub et al. 2007) and *Tetrahymena* (Harrington and Harrington 1991; Yu and Blackburn 1991) chromosomes. Stabilization of break points by telomere de novo synthesis was also found in the holocentric aphid *Myzus persicae* (Monti et al. 2011). In root meristems of maize, 50 % of the chromosome fragments caused by BFB cycles were stabilized after 3 weeks and up to 93 % after 10 weeks (Zheng et al. 1999). Similarly, in wheat, BFB cycles taking place after induced chromosome breakage were inhibited via adding telomeric sequences by telomerase to the break points (Tsujimoto 1993; Tsujimoto et al. 1997).

The lack of detectable amounts of newly formed telomeres and the differences in new telomeres signal intensities at some break points of *L. elegans* chromosomes might indicate a preferential binding of telomerase to specific repeats or other sequences at the fragment termini. Indeed, in *Saccharomyces cerevisiae*, the de novo telomere formation occurs preferentially at TG-rich sequences (Putnam et al. 2004). Additionally,

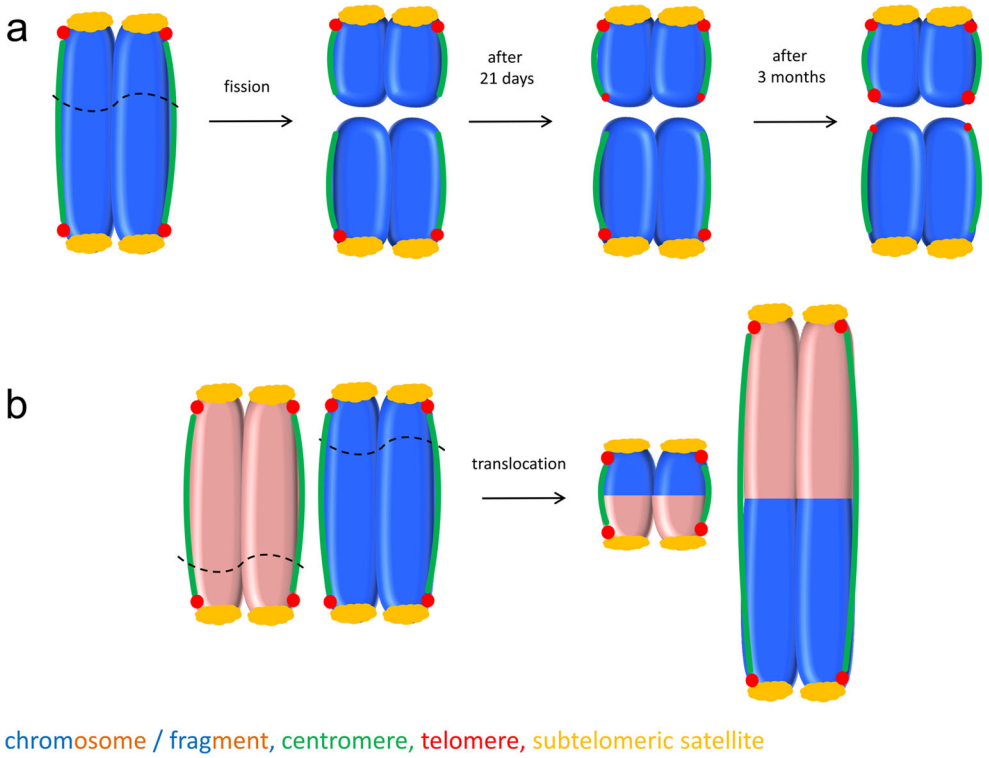

Fig. 5 Model illustrating possible karyotype alterations after fragmentation of holocentric chromosomes based on the interplay between holocentricity and telomere healing according to the behavior of *L. elegans* chromosomes. Irradiation of holocentric chromosomes induces chromosomal fragments of different size. Centromere activity is present along all chromosome fragments. Break points are indicated by *black dotted lines*. **a** Broken ends are negative for the terminal satellite probe and become gradually healed by de novo telomere syntheses, detected by a telomere-specific probe. **b** Translocations between non-homologous chromosomes form fragments of different size which are stabilized by pre-existing telomeres and the holocentromeres. Note that the distal location of LeSAT7 compared with the telomeres is likely caused by a fold back of telomere repeats (Heckmann et al. 2014)

the telomerase might preferentially extend shorter telomeres than longer ones causing different telomere signal intensities. The preferential elongation of shorter telomeres has been demonstrated in yeast (Teixeira et al. 2004), mouse (Hemann et al. 2001), and human (Britt-Compton et al. 2009). The occurrence of new telomeres displaying a similar FISH signal intensity as the pre-existing ones, as well as the presence of chromosomal fragments carrying telomere and LeSAT7 repeats at both ends, a short time after radiation, suggest the additional action of a telomerase-independent process such as terminal translocation or recombination. Simultaneous mechanisms of broken chromosome stabilization, de novo telomere synthesis by telomerase and telomere capture, were also reported in other species, e.g., human (Chabchoub et al. 2007).

Irradiation-induced fragments, translocations, and multivalent configurations do not impair the course of inverted meiosis in *L. elegans*. Similarly, no abnormalities in meiosis were detected after chromosome fragmentation in the holocentric *Cuscuta babylonica* and *R. pubera* species (Pazy and Plitmann 1994; Vanzela and Colaço 2002). The occurrence of multivalent configurations caused by irradiation was observed also in the holocentric plant *E. subarticulata* probably as the result of multiple translocations involving terminal heterochromatic region (Da Silva et al. 2005). The process of chromosome healing occurs gradually in *L. elegans*, and the cells have to pass several cell divisions to acquire a detectable number of telomeric repeats. All chromosome fragments present in the progeny of irradiated plants revealed telomeres at break points indicating their importance for fragment stabilization. In wheat, 2–4 weeks after chromosome fragmentation, stabilizing telomere repeats were observed only occasionally in root tip meristems, but during meiosis, all broken ends displayed cytologically detectable telomeres (Friebe et al. 2001).

The occasional occurrence of micronuclei in post-meiotic cells as well as the sporadic increase of the DNA amount in progeny plants might suggest missegregation of some chromosomal fragments during meiosis. Different factors may affect meiotic stability of fragments, e.g., fragment size, the presence of telomeres, and/or subtelomeric satellite repeats. A significant role of telomere repeats in chromosome end stabilization was demonstrated in the holocentric insect *Bombyx mori.* Here, fragments with telomeres at both ends were lost less often (25 %) during gametogenesis than fragments with telomeres present only at one end (56 %)

(Fujiwara et al. 2000). The importance of the terminal satellite repeats LeSAT7 and LeSAT11 during the inverted meiosis of *L. elegans* was previously demonstrated in (Heckmann et al. 2014). Additionally, other mechanisms, such as inappropriate attachment of spindle microtubules, kinetochore damage, and defects in the cell cycle control system might contribute to micronuclei formation (Luzhna et al. 2013).

In summary, we demonstrate that the combination of a holokinetic chromosome structure and the rapid formation of new telomeres at break points enable chromosome fragments to be successfully transmitted through mitotic and meiotic cell divisions. Thus, holocentric species may undergo a rapid karyotype evolution involving chromosome translocations and fissions (Fig. 5).

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