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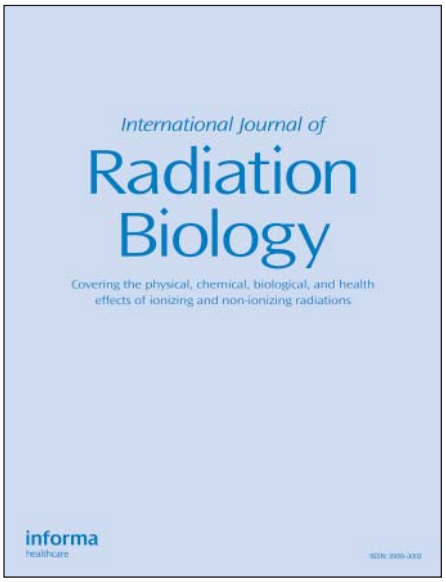

Efficiency of cytogenetic methods in detecting a chromosome rearrangement induced by ionizing radiation in a cultivated chili pepper line (*Capsicum baccatum* var. *pendulum* - SOLANACEAE)

Marisel A. Scaldaferro, Mauro Grabiele, J. Guillermo Seijo, Humberto Debat, M. Victoria Romero, Daniel A. Ducasse, Alberto R. Prina, Eduardo A. Moscone

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Abstract

Purpose: The present study was designed to locate transient chromosome aberrations on a selected pepper cultivar and determine the tracing efficiency of different cytogenetic methods.
Materials and methods: Seeds from *Capsicum baccatum* var. pendulum cultivar "Cayenne" were treated with an acute dose of X-rays (300 Gy) and chromosome aberrations were analysed by different cytogenetic methods [Feulgen, silver staining for nucleolus organizer regions (silver positive nucleolus organizing regions or AgNOR), fluorescent banding, fluorescence *in situ* hybridization (FISH) and meiotic analysis].
Results: A rearranged chromosome carrying two nucleolus organizing regions (NOR) induced by ionizing radiation was detected in the cultivar, with the occurrence of a small reciprocal exchange between a chromosome of pair no. 1 and another chromosome of pair no. 3, both carrying active NOR in short arms and associated chromomycin A positive / diamidino - phenylindole negative (CMA+/DAPI-) heterochromatin. Meiotic analysis showed a quadrivalent configuration, confirming a reciprocal translocation between two chromosomes.
Conclusions: The use of X-rays in *Capsicum* allowed us to develop and identify a pepper line with structural rearrangements between two NOR-carrying chromosomes. We postulate that all the cytological techniques employed in this research were efficient in the search for chromosome aberrations. Particularly, Feulgen and AgNOR were the most suitable in those cases of transient rearrangements, whereas fluorescent banding and FISH were appropriate for intransitive ones.

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Short title: Chromosome rearrangement by X-rays in *Capsicum*

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research were efficient in the search for chromosome aberrations. Particularly, Feulgen and AgNOR were the most suitable in those cases of transient rearrangements, whereas fluorescent banding and FISH were appropriate for intransitive ones.

Keywords: *Capsicum*, ionizing radiation, chromosome aberrations, cytogenetic

Introduction

Capsicum L. (tribe Solaneae, subtribe Capsicinae) ("chili pepper") is an economically important American genus including five domesticated species that are cultivated in tropical and temperate regions. They are currently used for human consumption as vegetables ("sweet pepper") and spices ("hot peppers"); they also have medical and ornamental value (Heiser 1995; Hunziker 2001).

Experimentally induced mutations in plants have broad applications, not only in basic genetic research but also in improvement programs for major crops (Prina 1989; Ahloowalia and Maluszynski 2001). During the past 70 years, radiation-induced mutations were the method most frequently used to develop direct mutant varieties (89%) and the use of chemical mutagens was relatively infrequent. With respect to physical mutagens, 64% of the radiation-induced mutant varieties were developed by gamma rays, whereas X-rays produced the remaining 22% (Ahloowalia et al. 2004).

In *Capsicum*, the effect of ionizing radiation and chemical mutagens has been frequently studied to estimate radio-sensitivity of main cultivars, production of mutants for breeding programs, increase of mutant allele gene bank and linkage group analyses (Saccardo and Sree Ramulu 1977; Daskalov 1986; Jabeen and Mirza 2004; Scaldaferro et al. 2004, 2013; Paran et al. 2007).

In chili peppers, most of the contributions about induced mutagenesis are focused on gene mutations; studies about changes at the chromosome level are scarce and most of them deal with polyploid induction (Indira and Abraham 1977; Katiyar 1977, 1978; Kumar and Raja Rao 2003). However, ionizing radiation is widely recognized as an effective method to induce chromosome structural changes, particularly reciprocal translocations (Gaul 1977). Accordingly, with the aim of developing chromosome structural rearrangements (i.e., reciprocal translocations), which would be valuable cytogenetic markers in chromosome pairing studies and genetic linkage in the genus, we applied ionizing radiation (X-rays) in *Capsicum*, with *C. baccatum* var. *pendulum* cv. "Cayenne" as a model plant species. Detailed karyotype and meiotic analyses were performed to examine the structural changes induced in second generation plants (M_2). Finally, we evaluated the tracing effectiveness of the different cytological methods employed for detecting pepper lines carrying rearranged chromosomes.

Materials and methods

Plant material

Dry seeds (500) of *Capsicum baccatum* L. variety *pendulum* (Willd.) Eshbaugh cultivar "Cayenne" (voucher specimen: E. A. Moscone and R. Neumann no. 211; Salta, Salta Province, Argentina) were treated with an acute dose of X-rays (300 Gray, Gy), according to the dose range reported for peppers in previous mutation experiments (Daskalov 1986; Scaldaferro et al. 2013). Irradiation was carried out on dormant seeds at 120Kv and 15mA and

room temperature. Dose rate of the X-ray machine was 67.8 R/sec (Roentgen/second) (0.65 Gray/second), at 29.0 cm distance (Philips MG 160 Constant Potential X-Ray System, Hamburg, Hamburg State, Germany).

The analysed cultivar has 2n = 2x = 24 and a normal karyotype with 11 metacentric (m) + 1 subtelocentric (st) pairs, 4 pairs (chromosomes 1, 3, 10, 12) carrying nucleolus organizer regions (NOR) and associated satellites in short arms (Levan et al. 1964; Moscone et al. 2007). The irradiated seeds were germinated in a growth chamber on filter paper in Petri dishes at 27/18ºC (day/night). Seedlings were transplanted to pots with soil and maintained in a glasshouse for 6 months to obtain the next generation (flowering and fruition). M_1 to M_4 plants from 1st to 4th generation were grown in a glasshouse under controlled light (12 h), temperature (25ºC) and humidity (40%) conditions.

Chromosome preparation

Somatic and meiotic chromosomes of M_2 seedlings were examined; these seedlings originated from the only M_1 surviving plant after the treatment of dry seeds with 300 Gy. In addition, mitotic metaphases of non-irradiated control lines were checked. For somatic chromosomes analyses, root tips (5-10 mm long) were collected and pre-treated with *p*-dichlorobenzene-saturated solution in the dark at room temperature for 2 h and then fixed in a freshly prepared 3:1 mixture (ethanol: glacial acetic acid) at 4ºC for a minimum of 12 h and stored at 20ºC until use. For meiotic chromosome study, flower buds were fixed and preserved in the same way. Chromosome spreads for AgNOR, fluorescent banding and fluorescence *in situ* hybridization (FISH) were performed after digestion of the material with enzymes [2% cellulase (weight/volume, w/v) (Serva, Heidelberg, Baden-Wurtemberg State, Germany), 1% pectinase (volume/volume, v/v) at 37ºC for 40 minutes (Sigma, Munich, Baviera State, Germany)] (Schwarzacher et al. 1980). The meristems were squashed in a drop of 45% acetic acid and, after removal of the coverslip with CO_2, slides were air dried, aged for 1-2 days at room temperature and stored at 20ºC until use. Meiotic observations were made in pollen mother cells (PMC), obtained from young anthers. Meiotic chromosomes were analysed in diakinesis-anaphase I.

Cytogenetic methods

Conventional Feulgen's staining method (Jong 1997) was applied to somatic chromosomes and meiotic chromosomes were dyed with acetic carmine (Figures 2A-C, 5A-B). Silver impregnation to detect NOR was performed after the silver-incubation procedure (Ag-I) (Bloom and Goodpasture 1976), setting the slides flooded with the Ag solution in moisture-tight plastic container, with modifications of Kodama et al. (1980), using nylon cloth (mesh size 0.243 mm) instead of coverslips (Figure 2D-F). Fluorescent chromosome banding to reveal type and distribution of constitutive heterochromatic regions was done according to the triple staining technique with the fluorochromes chromomycin A3 (Sigma), distamycin A (Sigma) and 4-6-diamidino-2-phenylindole (Sigma) (CMA/DA/DAPI) (Schweizer and Ambros 1994) (Figure 3). The symbols "+" or "-" were used to indicate increased or decreased fluorescence, respectively. FISH with 18-25S rDNA (ribosomal DNA) was

performed (Figure 4), using the 18 rDNA probe pCf18S-17 from *C. frutescens* (Grabiele et al. unpublished). The probe was labelled with biotin-11-dUTP (Sigma). Pre-treatment of slides and probe denaturation, conditions for *in situ* hybridization, post-hybridization washings, blocking, and indirect detection by fluorochrome conjugated antibodies [anti-biotin conjugated with TRITC (tetramethyl-rhodamine isothiocyanate, Dakopatts no. R270, Glostrup, Hovedstaden Region, Denmark) (red)] were performed as previously described (Moscone et al. 1996a).

Microscopy and image acquisition

Metaphases and meiotic chromosomes were observed and photographed, depending on the procedure, with transmitted light or epifluorescence using a Leica DMLB microscope equipped with the appropriate filter sets and a Leica DC250 digital camera (Leica, Heerbrugg, St. Gallen Canton, Switzerland). For epifluorescence microscopy, images were captured in black and white using appropriate filter sets. Yellow and blue images from triple staining CMA/DA/DAPI were captured in black and white using E3 and A filters (Leica) for CMA and DAPI. Red and blue images from FISH were captured in black and white using N2.1 and A filters (Leica) for TRITC and DAPI, respectively. Digital images were pseudo-coloured using IM 1000 Leica software, then imported into Photoshop 7.0 (Adobe, San Jose, California State, USA) for final processing.

Karyotype analysis

The chromosome nomenclature used was that of Levan et al. (1964). The idiogram (Figure 6) was based on chromosome measurements of fluorochrome stained metaphases (Moscone et al. 1996b).

Results

Capsicum baccatum var. *pendulum* cv. “Cayenne” (Figure 1), with 2n = 2x = 24, has a normal karyotype of 11 metacentric (m) + 1 submetacentric (sm) pairs, 4 pairs (chromosomes 1, 3, 10, 12) carrying nucleolus organizing regions (NOR) and associated satellites in the short arm (Figures 2A-D, 3A-B, 4A, 6A). M_2 seedlings originated from the only surviving M_1 plant, following the 300 Gy treatment, had a rearranged metacentric chromosome carrying active NOR in both arms (Figure 2B-F). The 300 Gy treatment had been selected previously because it was demonstrated to produce the highest number of chromosome aberrations in M_1 (Scaldaferro et al. 2004, 2013). The abnormality was studied with conventional staining techniques: Feulgen's method, AgNOR (Figure 2), fluorescent banding (Figure 3) and FISH (Figure 4). At first, the recognition of the chromosome with two satellites was made with classic staining technique (Figure 2B-C). Such change became an easily recognizable feature, despite the small NOR size of *C*. *baccatum* var. *pendulum*.

The screening of 36 M_2 seedlings showed that 44% of them were heterozygous for the exchange (Figures 2B-E, 3C-D, 4B, 6B), 8% were homozygous (Figures 2C-F, 3E-F, 4C, 6B), and the remaining individuals were normal homozygous (Figures 2A-D, 3A-B, 4A, 6A). The lack of chromosomal

instability observed indicates the occurrence of a small reciprocal exchange between a member of chromosome pair no. 1 and another member of chromosome pair no. 3, both carrying active NOR in short arms and associated CMA+/DAPI- heterochromatin, according to the normal cultivar karyotype (Figure 6A-B). The results of this structural rearrangement were two chromosomes with little modification in size; one of them, chromosome 3, was easily recognizable by the presence of NOR and CMA+/DAPI- associated heterochromatin in both arms, designated hereafter as chromosome 3_1, whereas the other chromosome (no. 1), hereafter chromosome 1_3, of difficult identification, bears a small heterochromatic CMA+/DAPI- band in the short arm.

After that, AgNOR method was applied to chromosome preparations, and the presence of translocation was confirmed, both in heterozygous and homozygous state (Figure 2E-F). In M_2 seedlings, the number of AgNOR in metaphase and the maximum number of nucleoli in interphase nuclei were counted. In this translocated line, the number observed in the latter was always lower than in the former. Interphases with 7 or 8 nucleoli were never observed, whereas up to 40% of metaphases with 8 AgNOR were found, (20%) 7, (10%) 6, (10%) 5, and (20%) 4. In addition, signals obtained by FISH showed the same pattern of translocation, which is consistent with the previous findings from this study (Figure 4).

Meiotic analysis of 29 pollen mother cells (PMC) at diakinesis from five seedlings of the M_2 generation demonstrated the presence of a ring quadrivalent configuration (10 II + 1 IV) in addition to the expected 12 II, 38% vs. 62%, respectively (Figure 5A-B). Further analysis on seed germination showed that, notwithstanding normal seed production in all the segregated plant fruits, they produced only 52.05% percentage of germination.

At present, M_3 and M_4 generations of translocated line are being grown in a glasshouse (Figure 1) for further analyses to assess the permanence of translocation and to check the productivity in subsequent generations.

Discussion

In the present study, the use of X-rays in *Capsicum* seeds allowed our group to develop and identify a pepper line with a structural rearrangement between two NOR-carrying chromosomes. A rearranged chromosome emergence bearing NOR in both arms, which is a result of an induced reciprocal translocation as described here for pepper, was also observed in barley (Prina 1989). In that work, the meiotic study in M_2 revealed that cytological aberrations were due to heterozygous reciprocal translocations, and approximately 20% of reciprocal translocations were easily recognizable in mitosis, yielding chromosomes with a different morphology than the normal karyotype.

In M_2 “Cayenne” seedlings, the phenomenon of intrachromosomal nucleolar dominance was observed. The number of AgNOR in metaphase did not match the maximum number of nucleoli in interphase nuclei, the latter value being always lower. Interphases with 7 or 8 nucleoli were never detected. The lack of correlation between the number of AgNOR in metaphase and the maximum number of nucleoli in interphase nuclei was probably due to a

nucleolar association with fusion during interphase (Nicoloff et al. 1977, 1979; Sato et al. 1981), and to the possibility of occurrence of intrachromosomal nucleolar dominance, in which nucleolus organizers on the same chromosome pair compete in nucleolus formation. However, in a study of transcriptional activity of an inversion split NOR in barley, both parts of the split NOR proved to be transcriptionally active, and were not subject to intrachromosomal nucleolar dominance (Georgiev et al. 2001).

Chromosome aberrations are a very sensitive biological endpoint reflecting the consequences of wrong or lack of repair of initial DNA lesions. The two principal types of chromosome aberrations induced by ionizing radiation are the classical intransitive aberrations, including mainly dicentromic chromosomes and acentric fragments and rings, and the transient ones, such as translocations and insertions. Most of chromosome symmetrical exchanges cannot be observed through conventional staining methods; nevertheless, they can be identified by using chromosome banding techniques that facilitate visualization. The advent of “banding” in mammalian chromosomes providing morphological markers along chromosome arms allowed the detection of symmetric chromosome exchanges, i.e., translocations (Bigger et al. 1972).

In this study, the exchange was assessed with four cytological techniques, i.e., Feulgen, AgNOR, fluorescent banding and FISH (Figures 2-4), showing that Feulgen and AgNOR methods (Figure 2) were the most informative in the inquiry of these structural changes. However, triple staining with fluorochromes also proved to be a helpful tool for the scoring of chromosome aberrations (Figure 3). Another powerful tool used in the current study was FISH (Figure 4) shown to be a very effective method for detecting both transient and intransitive chromosome aberrations (Lucas et al. 1992; Natarajan et al. 1992; Schmid et al. 1992; Boei et al. 1994). This method allowed us to confirm the reciprocal translocation between two chromosomes bearing NOR (Figure 4B-C). We postulate the usefulness of all these cytological techniques in the search of chromosome aberrations. Particularly, Feulgen and AgNOR are the most efficient methods in those cases where there have been insertions or reciprocal translocations. Likewise, fluorescent banding or FISH are proposed in the case of intransitive aberrations, like incomplete chromosome elements and interstitial fragments, according to previous studies (International Atomic Energy Agency 2001; Bolzán and Bianchi 2004). In this study, Feulgen and AgNOR were the most informative techniques because they facilitated NOR translocated visualization (Figure 2). In FISH and chromosome banding fluorescence, observation was difficult; in addition, these techniques were not so informative, because of the karyotype features of *C. baccatum* var. *pendulum*, particularly characterized by the presence of several small terminal bands (Figures 3, 4 and 6).

The meiotic analysis confirmed the exchange that had been suggested previously on somatic metaphases (Figure 5B). The observed segregation could be explained in terms of genetic constitution of the chimeric M_1 parent plant of the translocated line. As the mutagenic treatment was performed on multicellular material (seeds) in the first generation plant undergoing mutational

changes at each individual cell level, cells of different tissues could be mutated in different directions. M_{2-4} pepper plants carrying the translocation described above do not show any distinctive phenotype (Figure 1A-B), although some experiments in barley have demonstrated that certain lines, homozygous for translocated chromosomes, are superior to their chromosomally normal mother varieties (Gustafsson et al. 1966, 1971; Hagberg and Hagberg 1971; Hagberg et al. 1972; Künzel and Scholz 1972).

Another particular feature was the normal seed set production, which is not in accordance with the mutation type found here, since a fertility reduction is usually expected in heterozygous reciprocal translocation. This decrease is due to chromosome non-balanced segregation of the quadrivalent in anaphase I (alternative or adjacent, 1:1), according to convincing data provided by Ramage (1960). Hence, in each case the heterozygous forms should display a visible reduction in seed set production. Despite the normal production of the M_2 seeds in all the segregated plant fruits, their reduced percentage of germination confirmed the presence of the quadrivalent configuration. In maize, the heterozygous exchanges, which mostly form rings in anaphase, are often approximately 50% sterile, suggesting that the “open” and “zigzag” rings may be equally frequent (Burnham 1962). Most of the exchanges in plants are normal when homozygous, in contrast to the behaviour in animals, probably due to the presence of a gametophyte screen in higher plants against deficiencies, which is absent in animals (Burnham 1962). Therefore, this translocated line can be maintained carrying a valuable cytogenetic marker.

Irradiation with 300 Gy was almost lethal according to a previous dosimetric analysis (Scaldaferro et al. 2004, 2013). Despite such lethality, our research group achieved a pepper line in *C. baccatum* var. *pendulum* cv. “Cayenne” carrying a valuable induced translocation after applying that dose. The different cytogenetic techniques we have used proved to be effective in the search of particular chromosome aberrations. In addition, the identified chromosome marker, i.e., the presence of NOR and associated satellites in both arms, is an easily recognizable feature and, therefore, it is considered appropriate for gene mapping, particularly for those genes with agronomic value in the selected cultivar.

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Declaration of interest

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Figure Legends

Figure 1

M_4 plants of *Capsicum baccatum* var. *pendulum* cv. “Cayenne” from the 300 Gy X-ray treated line with a rearranged chromosome bearing NOR in both arms in homozygous condition, which display a normal phenotype. **A-B** Different seedlings growing in pots coming from the same M_3 plant. **C** Branch with flower. **D** Immature (green, left) and mature fruits (orange, right). In **A** and **B** bar represents 10 cm; in **C** and **D** bar represents 2 cm.

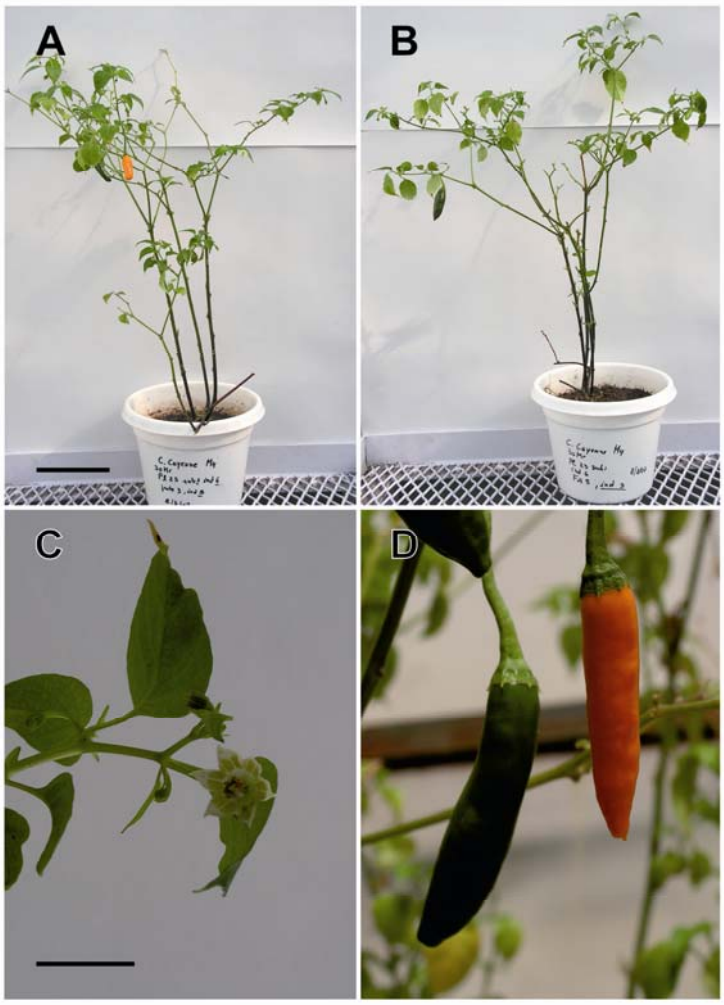


Fig. 1

Figure 2

Feulgen (**A-B-C**) and silver stained (**D-E-F**) somatic metaphases of *Capsicum baccatum* var. *pendulum* cv. "Cayenne" (2n = 24). **A** and **D** Control (non-irradiated) line plant showing four pairs of nucleolus organizing regions (NOR). **B-F** 300 Gy-treated line plants of the M_2 generation exhibiting one reciprocal translocation that affects a NOR. In **B-E** plant heterozygous for the exchange. In **C-F** plant homozygous for the exchange. Normal NOR-bearing chromosomes are indicated with the same number as in the idiogram of Fig. 6**A**. The rearranged chromosome carrying NOR in both arms is indicated according to the identification number of the involved original chromosomes as represented in Fig. 6**B**. Arrowheads point NOR. Bars represents 10 µm, bar in **C** is for **A**, **B** and **C**, and bar in **F** is for **D**, **E** and **F**.

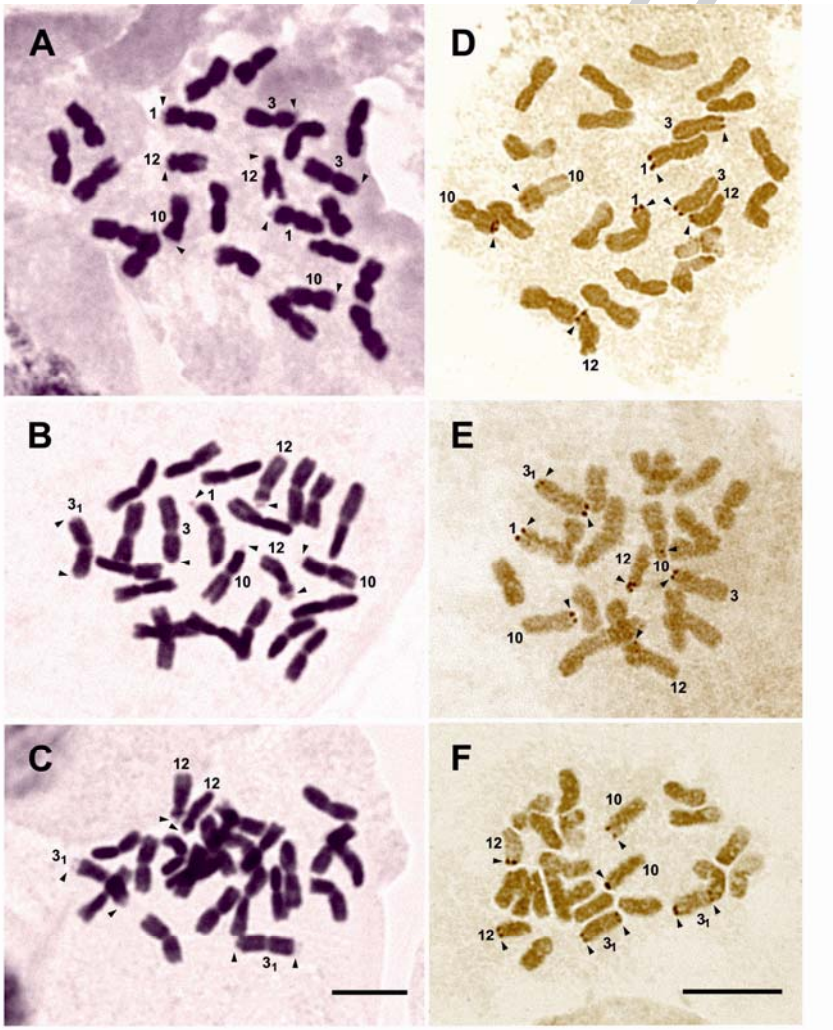


Fig. 2

Figure 3

Somatic metaphases of *Capsicum baccatum* var. *pendulum* cv. "Cayenne" (2n = 24) triple stained with CMA/DA/DAPI, which show CMA+/DAPI- heterochromatic bands. **A-C-E** CMA/DA fluorescence. **B-D-F** DA/DAPI fluorescence. **A-B** Control (non-irradiated) line plant displaying four chromosome pairs with NOR-associated heterochromatin. **C-F** 300 Gy-treated line plants of the M_2 generation exhibiting one reciprocal translocation which affects a NOR. In **C-D** plant heterozygous for the exchange. In **E-F** plant homozygous for the exchange. In **A**, the homologous chromosomes are identified with the same numbers as in the idiogram of Fig. 6**A**. In **C** and **E**, normal NOR-bearing chromosomes are indicated with the same numbers as in the idiogram of Fig. 6**A**, and rearranged chromosomes, with the same numbers as in Fig. 6**B**. Arrowheads point CMA+/DAPI- NOR-associated heterochromatin. Bar represents 10 µm.

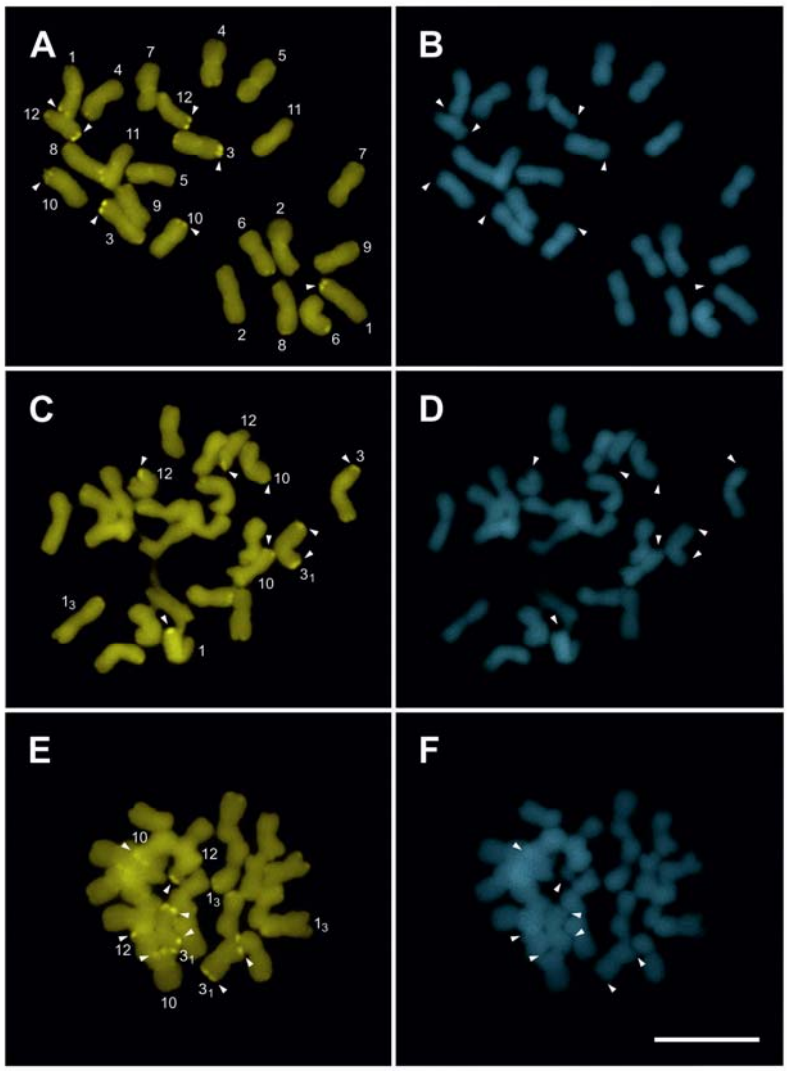
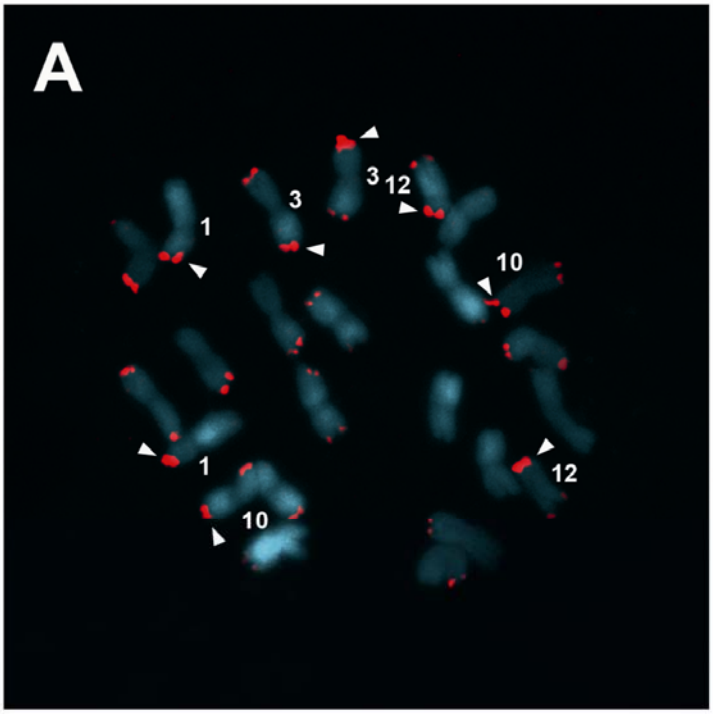
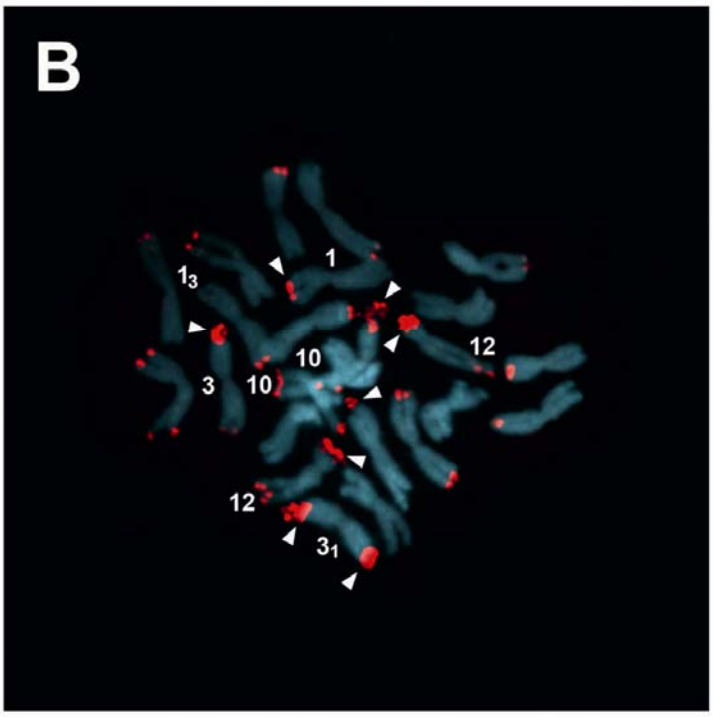


Fig. 3

Figure 4

FISH to somatic metaphases of *Capsicum baccatum* var. *pendulum* cv. "Cayenne" (2n = 24) using the 18S-25S rDNA probe detected with TRITC (red, brigthness), the chromosomes are counterstained with DAPI (blue, soft shine). **A** Control (non-irradiated) line plant showing rDNA signals in the four pairs of chromosomes with active NOR (chromosomes 1, 3, 10, 12). **B** 300 Gy-treated line plant of the M_2 generation exhibiting a reciprocal translocation between the chromosome pairs no. 1 and 3 affecting one NOR, heterozygous for the exchange. **C** Plant homozygous for the same exchange. Arrowheads point active NOR. Bar represents 10 µm.





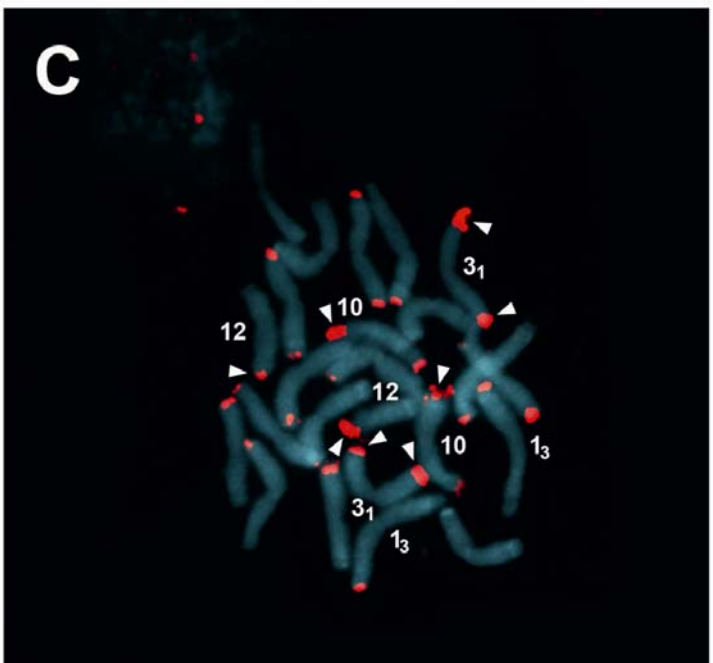


Figure 5

Acetic carmine stained pollen mother cells at diakinesis. **A** Control (non-irradiated) line plant showing 12 bivalents. **B** M_2 heterozygous plant of the 300 Gy X-ray treated line showing 10 bivalent plus one quadrivalent ring. Bar represents 10 µm.

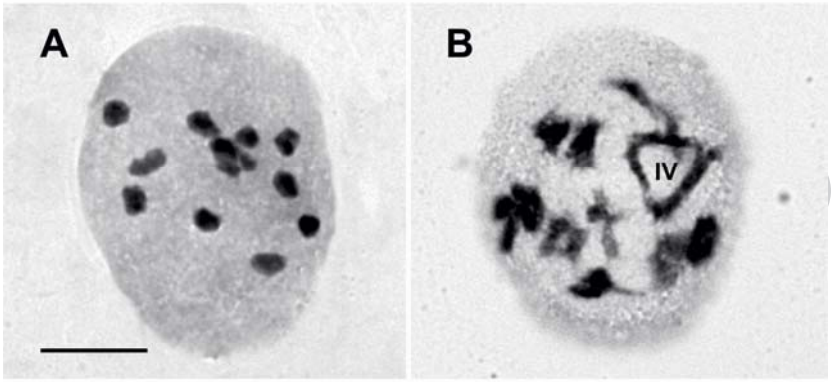


Fig. 5

Figure 6

A Idiogram of *Capsicum baccatum* var. *pendulum* cv. "Cayenne" showing the heterochromatic fluorochrome banding pattern and four nucleolus organizing regions. **B** Original chromosomes involved in a reciprocal translocation induced by X-ray, and the respective rearranged chromosomes as a result of the exchange. Asterisks indicate the position of the exchanged regions. Solid blocks indicate CMA+/DAPI- heterochromatic bands. Bar represents 5 µm.

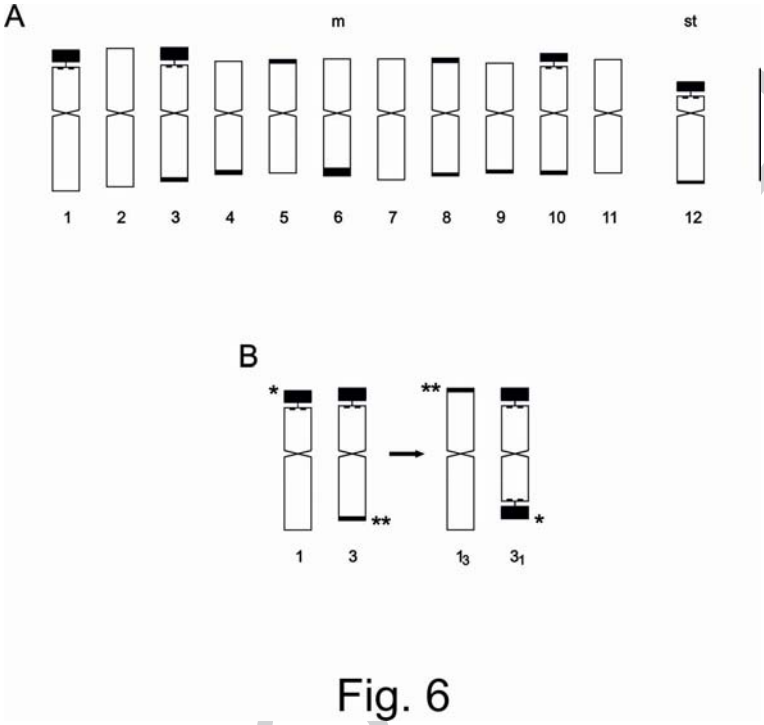


Fig. 6