No evidence for “break-induced replication” in a higher plant – but break-induced conversion may occur

Ingo Schubert*, Veit Schubert and Jörg Fuchs

Department of Cytogenetics and Genome Analysis, Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany

Edited by: Richard Jorgensen, Laboratorio Nacional de Genomica para la Biodiversidad, Mexico.
Reviewed by: Paul Franz, University of Amsterdam, Netherlands
Charles I White, National Center for Scientific Research, France
Alan Christensen, University of Nebraska, USA

*Correspondence:
Ingo Schubert, Leibniz Institute of Plant Genetics and Crop Plant Research, Corrensstrasse 3, D-06466 Gatersleben, Germany.
e-mail: schubert@ipk-gatersleben.de

INTRODUCTION

Double-strand breaks (DSBs) are the most critical DNA lesions. Unrepaired DSBs are usually lethal for dividing cells due to the subsequent loss of the acentric fragment and the unstable break-end of the centric fragment. DSBs can be caused enzymatically, by ionizing irradiation, or by other S phase-independent clastogens. DSBs with only one break-end may appear when a replication fork meets a single-strand break or a repair-mediated parental single-strand discontinuity.

There are several ways to repair DSBs. In case of a restriction endonuclease-mediated break a simple ligation can restore the pre-break situation. Mostly however, the break is extended by a 5′-specific exonuclease activity generating 3′-overhanging ends. As soon as overhanging ends meet at short stretches of complementary bases (single-strand annealing, SSA), the distal free ends are resected, and the break becomes ligated, resulting in a (micro-)deletion (Figure 1A). Alternatively, the break-ends may invade undamaged homologous template strands and a limited DNA synthesis across the break region is followed by re-ligation of both strands (synthesis-dependent strand annealing, SDSA). The result is genetically not detectable when the template was the undamaged sister chromatid; it appears as a gene conversion if an allele of the homologous chromosome served as a template (Figure 1C). If for break-end elongation an ectopic or an extra-chromosomal (partially) homologous sequence is used, then, depending on the template double helix involved, a sister chromatid exchange, a crossing over, or a reciprocal translocation results.

“Break-induced replication” (BIR) is considered as one way to repair DNA double-strand breaks (DSBs). BIR is defined as replication of the proximal break-ends up to the end of the broken chromosome using an undamaged (homologous) double-stranded template and mimicking a non-reciprocal translocation. This phenomenon was detected by genetic experiments in yeast. BIR is assumed to occur also in mammals, but experimental evidence is not yet at hand. We have studied chromosomes of the field bean, Vicia faba L., with respect to the occurrence of BIR after DSB induction during S and G2 phase. Simultaneous incorporation of the base analog ethynyldeoxyuridine (EdU) revealed no chromosomal replication pattern indicative of BIR. Thus, if occurring at all, BIR does not play a major role in DSB repair in higher plants with large chromosome arms. However, the frequency of interstitial asymmetric EdU incorporation within heterodromatic regions, visible on metaphase chromosomes, increased after chromosome breakage during S and G2 phase. Such asymmetric labeling could be interpreted as conservative replication up to the next replicon, circumventing a DSB, and yielding an interstitial conversion-like event.

Keywords: break-induced replication, Vicia faba, chromatid aberrations, cell cycle, base analog, non-conservative replication, chromosome labeling, double-strand break
Materials and Methods

The field bean, *Vicia faba* L., karyotype ACB with six large and individually distinguishable chromosome pairs (Döbel et al., 1978) is much more comfortable for aberration scoring than for instance *A. thaliana* with smaller chromosomes and less mitoses per root meristem.

Seeds were germinated for 3 days on wet filter paper at room temperature in the dark. About 1–2 cm long primary roots were incubated for 18 h in aerated Hoagland solution, pH 5.5, containing 1.25 mM hydroxyurea which synchronizes cells by blocking the cell cycle in early S phase. For mutagen treatment during S phase, the roots were incubated for 1.5 h in Hoagland solution containing 10 μg/ml bleomycin with or without 20 μM EdU (Invitrogen) followed by incubation in Hoagland solution for 4.5 h and then 0.05% colchicine for 2.5 h (to arrest metaphases) before fixation in ethanol:acetic acid (3:1) overnight. For scoring of chromatid aberrations, roots were washed for 10 min in distilled water, hydrolyzed for 11 min in 1 N HCl at 60°C, stained for 30–40 min in Feulgen solution and squashed in a drop of 45% acetic acid. Only complete metaphase cells were scored for chromatid aberrations: chromatid and isochromatid breaks (one or both sister chromatids with terminal deletion), interstitial deletions, duplication deletion (the deleted part of one chromatid is inserted into a break of the sister chromatid), and reciprocal chromatid translocations.

**FIGURE 1** Pathways of DSB misrepair via single-strand annealing (SSA) or via synthesis-dependent strand annealing (SDSA).

(A) Deletion via exonucleolytic 5’-end resection, SSA at complementary overhang sequences, resection of the non-aligned ends, and ligation of break-ends. (B) Insertion into a DSB by break-end invasion and elongation along an ectopic and partially homologous (vertical bars) template. (C) Re-synthesis of break-ends after invasion into a homologous template double-strand without (gene conversion) or with exchange of flanking regions due to appropriate resolution of Holiday junctions (green arrow heads).
For mutagen treatment during G2 phase, primary roots were incubated in Hoagland solution with hydroxyurea as above, then in Hoagland solution for 4.5 h and subsequently in Hoagland solution with bleomycin, with EdU, or both for another 1.5 h followed by 2.5 h in colchicine before fixation, staining, and evaluation.

RESULTS
BLEOMYCIN INDUCES CHROMATID ABERRATIONS IN S AND G2 PHASE; SIMULTANEOUS TREATMENT WITH THE BASE ANALOG EDU DOES NOT FURTHER INCREASE THE ABERRATION YIELD
The S phase-independent clastogen bleomycin induces DNA breaks directly. A dose of 10 μg/ml was previously shown to yield chromatid aberrations in up to 30% of the first post-treatment metaphases of field bean root tip meristems (Heindorff et al., 1987). The same bleomycin concentration applied for 1.5 h to (mainly) G2 cells of root tip meristems, in two independent experiments yielded on an average of 14.8% of metaphases with chromatid aberrations. After treatment during S phase 9.2% of aberrant metaphases were found. Treatment with EdU (20 μM, 1.5 h) resulted in 0.8% metaphases with aberrations when applied during G2 and in 3.1% when applied during S phase. Untreated control roots displayed 1% of metaphases with aberrations. Bleomycin and EdU together yielded 12% of metaphases with aberration after exposure in G2 and 6.6% after exposure to S phase cells (Table 1). Although during S phase the base analog EdU caused slightly more aberrations than appear in untreated control cells, no obvious synergistic effect on clastogenicity was observed when applied together with bleomycin.

CHROMOSOMAL EDU INCORPORATION PATTERNS DO NOT INDICATE BREAK-INDUCED REPlication UP TO AN ARM END
When root tips were incubated in 20 μM EdU 8.5–7 h before fixation, most metaphase chromosomes (87.4%) were completely labeled and some (12.5%) were labeled along their euchromatin regions (Table 2), indicating that the corresponding cells were in S phase during treatment as expected according to the experimental schedule based of the cell cycle duration of field bean meristems (Schubert and Meister, 1977). No chromosome showed asymmetric labeling up to the chromosome end, i.e., an unlabeled terminal region of one of the sister chromatids. Only one out of 1404 chromosomes displayed an unlabeled transversal region within a heterochromatic area of one sister chromatid.

When S phase cells were treated simultaneously with EdU and bleomycin, all 2130 observed chromosomes were labeled (81.4% completely and 18% along their euchromatin). None of these chromosomes showed asymmetric labeling up to an arm end as to be expected if the replication fork is stalled at a single-strand break and the 3’-break-end recurrently invaded the unreplicated double helix, or if it replicated till the end of the template by unidirectional fork migration (Figure 2A and Hastings et al., 2009).

Thirteen chromosomes showed unlabeled transversal spots within the heterochromatic part of one sister chromatid (Figure 3B). Since 2130 chromosomes correspond to 178 cells, in about 7.3% of cells such an asymmetrically unlabeled spot occurred, representing an eightfold increase compared to the variant with EdU treatment alone.

Alternatively, the root tips were fixed in 4% formaldehyde for 20 min for isolation of metaphase chromosomes (Schubert et al., 1993) and subsequent detection of EdU incorporation using the Click.iT EdU Imaging Kit from Invitrogen according to manufacturer’s instruction. Microscopic analysis of chromosomes was performed with an epifluorescence microscope (Zeiss axioskop) using a 100x/1.45 Zeiss alpha-plan-fluar objective and a Sony (DXC-950P) camera. Images of fluorescent chromosomes were captured using filter sets F36-513 for DAPI and F36-750 for Alexa Fluor 594 (AHF Analysentechnik, Germany). DAPI and Alexa Fluor 594 azide images were merged using Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA, USA).
**Table 1** | Chromatid-type aberrations induced by EdU, bleomycin, or both during G2 or S phase in field bean root tip meristems (summary of two independent experiments).

| Evaluated cells | Metaphases with aberrations (%) | t | i | d | b | DSB |
|-----------------|-------------------------------|---|---|---|---|-----|
| G2 EdU          | 250                           | 2 (0.8)       | – | 1 | – | 1 3 |
| G2 bleomycin    | 350                           | 52 (14.8)     | 5 | 7 | – | 46 70 |
| G2 EdU + bleomycin | 250                        | 30 (12.0)     | 4 | 1 | – | 27 37 |
| \( \Sigma \) bleomycin/EdU + bleomycin | 600                        | 82 (13.7)     | 9 | 8 | – | 73 107 |
| S EdU           | 350                           | 11 (3.1)      | 3 | 5 | – | 3 19 |
| S bleomycin     | 400                           | 37 (9.2)      | 9 | 24 | 2 | 6 76 |
| S EdU + bleomycin | 350                       | 23 (6.6)      | 7 | 4 | 5 | 8 40 |
| \( \Sigma \) bleomycin/EdU + bleomycin | 750                        | 60 (8.0)      | 16 | 28 | 7 | 14 116 |

\( t \) = reciprocal translocation, \( i \) = isochromatid break, \( d \) = interstitial deletion, \( b \) = chromatid break, see Figure 3A.

**Table 2** | Type and frequency of chromosomal labeling patterns after EdU incorporation during S or G2 phase with or without bleomycin treatment (two independent experiments summarized).

| Chromosomes evaluated | Completely labeled (%) | Late replication unlabeled | Asymmetric unlabeled* | Cells*** |
|-----------------------|------------------------|---------------------------|----------------------|---------|
| S                     |                        |                           |                      |         |
| EdU                   | 1404                   | 1227 (87.4)              | 175 (12.5)           | 1 (0.1) | 117 (0.9) |
| EdU + bleomycin       | 2130                   | 1734 (81.4)              | 383 (18.0)           | 13 (0.6) | 178 (7.3) |

| LATE S/G2            |                        |                           |                      |         |
|                       | Unlabeled              | Late replication labeled  | Asymmetric labeled** | Cells   |
| EdU                   | 2366                   | 1923 (81.3)              | 433 (18.3)           | 10 (0.4) | –197 (5.1) |
| EdU + bleomycin       | 1557                   | 1331 (86.5)              | 206 (13.2)           | 20 (1.3) | –130 (15.4) |

*see Figures 2A and 3B.* **see Figures 2B and 3C.*** in () maximum percentage of cells harboring an asymmetrically labeled chromosome.

When metaphase chromosomes were tested for EdU incorporation after incubation in EdU 4–2.5 h before fixation, the corresponding cells should have been in G2 during EdU treatment. Indeed, most of the chromosomes (81.3%) were unlabeled while 18.3% revealed late replication pattern (symmetric labeling of heterochromatic regions) thus indicating they were in late S phase during EdU treatment. No chromosome showed asymmetric labeling of one chromatid up to the arm end, as expected in the case of a conservative BIR (Figure 2B). Also symmetric terminal labeling of sister chromatids, as expected in the case of semiconservative BIR (Figure 2C) was not observed. Single transversal interstitial fluorescence signals on one of the sister chromatids (Figure 3C) were observed in 10 (0.4%) chromosomes.

After simultaneous exposure to EdU and to bleomycin 4–2.5 h before fixation, again most of the chromosomes (85.5%) were completely unlabeled and thus were in G2 during treatment; 13.2% of chromosomes showed late replication patterns, indicating that the corresponding cells were in late S during treatment. Again no chromosome showed asymmetric or symmetric terminal labeling as predicted for BIR. However, there was a 3-fold increase in chromosomes showing an interstitial transversal and asymmetric fluorescence signal (20 signals per 130 cells), usually in heterochromatic regions.

**DISCUSSION**

If BIR occurs in higher plants in the same way as postulated for yeast (i.e., as replication extending from the break position up to the corresponding chromosome arm end) conservative replication in S or G2 (Figures 2A,B) or semiconservative replication in G2 (Figure 2C) should be detectable. Of the chromatid aberrations observed after bleomycin and bleomycin plus EdU treatment during S and G2, reciprocal translocations (25), isochromatid breaks (36), and interstitial deletions (7) require two DSBs each, while simple chromatid breaks (87) representing terminal deletions, go back to a single DSB. Therefore, within 600 G2 and 750 S phase cells, bleomycin caused 223 DSBs which underwent misrepair. Thus, on average at least one DSB per six cells was induced. Considering that a fraction of DSBs might have been repaired via pathways not resulting in microscopically detectable chromatid-type aberrations (for instance by sister chromatid exchange, intrachromosomal SSA, or gene conversion), the actual number of DSBs may be higher. However, in a number of chromosomes corresponding to 308 S and G2 cells, treated simultaneously with the chromosome-breaking agent bleomycin and the base analog EdU, not a single chromosome showed a labeling pattern indicative of BIR. In budding yeast, more
than half of diploid cells repair HO-endonuclease-induced DSBs by BIR using the homologous chromosome as template (Bosco and Haber, 1998); meaning more than half of the DSBs are repaired via BIR if the endonuclease HO causes one DSB per diploid yeast cell. Had BIR occurred with a similar efficiency in the field bean, we should have observed >223 instead of no chromosomes with asymmetric terminal EdU signal. Our result does not exclude the occurrence of BIR in plants, however, the frequency of such events is below 0.45% (less than 1 out of 223) of the DSBs causing chromosome structural aberrations, i.e., more than 100-times lower than in yeast. Unidirectional replication forks that override distal replication origins have to migrate up to the arm ends, or, alternatively, the requirement of recurrent invasion of 3′-ends into other double helices, are presumptions for which experimental evidence is lacking so far. Long range replication in addition to the regular round of replication between two nuclear divisions, represent a further unproven assumptions. To our knowledge no experimental evidence as to the occurrence of BIR in animals has been shown (Zhang et al., 2009). Taken together these arguments imply that the existence of a BIR pathway in higher eukaryotes is unlikely. Even when such events occur, their frequency is much lower than in yeast arguing against their importance as a pathway for DSB repair at least in higher plants such as the field bean. Because the 12 megabase pair (Mb) genome size of *Saccharomyces cerevisiae* is distributed over 32 chromosome arms, the risk that BIR interferes with replication licensing at the replicon level is much smaller than for species with orders of magnitude larger genomes with several Mb per arm. Also complex chromosome rearrangements (e.g., Difilippantonio et al., 2002; Zhang et al., 2009) can more easily be explained by other well-documented pathways (Zhang et al., 2009; Schubert and Lysak, 2011) rather than by BIR via recurrent invasion of a DSB-induced 3′-end into different template double helices. The more so as experimental approaches even in yeast cannot differentiate “between BIR and gene conversion events involving G2 cross over, followed by appropriate chromatid segregation” (Ricchetti et al., 2003).

Nevertheless, the increased frequency of interstitial asymmetric labeling patterns of chromosomes exposed to bleomycin and EdU during S or G2 might indicate that conserved replication, although not reaching arm ends, could be linked with DSB repair to result in microscopically detectable stretches of gene conversion extending hundreds of kilobases (possibly, up to the junction with the next replication unit, but not covering DNA stretches of >1 Mb up to the arm ends). Such extended gene conversion-like repair might be considered as BIR, if the postulation “up to the chromosome terminus” is excluded. Similar events could potentially have been responsible for the “zebra” structure of the long arm of chromosome z5A observed in a backcross progeny between *Elymus trachycaulus* Gould ex Shinners and *Triticum aestivum* L. (Zhang et al., 2008).
The preferential appearance of local asymmetric labeling within heterochromatic regions of the field bean suggests that for late replicating repetitive DNA the units of replication regulation are larger or more variable than for euchromatin.

REFERENCES

Bosco, G., and Haber, J. E. (1998). Chromosome break-induced DNA replication leads to nonreciprocal translocations and telomere capture. Genetics 150, 1037–1047. Dililippantonio, M. J., Petersen, S., Chen, H. T., Johnson, R., Jasins, M., Kanaar, R., Ried, T., and Nussenzweig, A. (2002). Evidence for replicative repair of DNA double-strand breaks leading to oncogenic translocation and gene amplification. J. Exp. Med. 196, 469–480. Döbel, P., Schubert, I., and Riegler, R. (1978). Distribution of heterochromatin in a reconstructed karyotype of Vicia faba as identified by banding and DNA-late replication patterns. Chromosoma 69, 193–209. Fuchs, J., Pich, U., Meister, A., and Haber, J. E. (1999). DNA recombination: Heterechromatin in the origin of human copy number variation. Trends Biochem. Sci. 24, 271–275. Haber, J. E. (1999). DNA recombination: the replication connection. Trends Biochem. Sci. 24, 25–28. Haber, J. E. (2006). Transpositions and translocations induced by site-specific double-strand breaks in budding yeast. DNA Repair 5, 998–1009. Hastings, P. J., Ira, G., and Lupski, J. R. (2009). A microhomology-mediated break-induced replication model for the origin of human copy number variation. PLoS Genet. 5, e1000327. doi: 10.1371/journal.pgen.1000327

Heindorff, K., Schubert, I., Riegler, R., and Michaelis, A. (1987). Cytogenetic adaptation of Vicia faba root-tip meristem cells after consecutive treatments with S-phase dependent and S-phase independent agents. Biol. Zent. Bl. 106, 439–448. Kotogoy, E., Dudits, D., Horvath, G. V., and Ayaydin, F. (2010). A rapid and robust assay for detection of S-phase cell cycle progression in plant cells and tissues by using ethynyl deoxyuridine. Plant Methods 6, 5. Lorente, B., Smith, C. E., and Symington, L. S. (2008). Break-induced replication: what is it and what is it for? Cell Cycle 7, 859–864. Malkova, A., Ivanov, E. L., and Haber, J. E. (1996). Double-strand break repair in the absence of RAD51 in yeast: a possible role for break-induced DNA replication. Proc. Natl. Acad. Sci. U.S.A. 93, 7131–7136. Malkova, A., Naylor, M. L., Yamaguchi, M., Ira, G., and Haber, J. E. (2005). RAD51-dependent break-induced replication differs in kinetics and checkpoint responses from RAD51-mediated gene conversion. Mol. Cell Biol. 25, 933–944. McEachern, M. J., and Haber, J. E. (2006). Break-induced replication and recombinational telomere elongation in yeast. Annu. Rev. Biochem. 75, 111–135. Morrow, D. M., Connelly, C., and Hieter, P. (1997). “Break copy” duplication: a model for chromosome fragment formation in Saccharomyces cerevisiae. Genetics 147, 371–382. Puchta, H. (2005). The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. J. Exp. Bot. 56, 1–14. Ricchetti, M., Dujon, B., and Fairhead, C. (2003). Distance from the chromosome end determines the efficiency of double strand break repair in subtelomeres of haploid yeast. J. Mol. Biol. 328, 847–862. Schubert, I., Dolezel, J., Houben, A., Scherthan, H., and Wanner, G. (1993). Interpreted examination of plant metaphase chromosome structure at different levels made feasible by new isolation methods. Chromosoma 102, 96–101. Schubert, I., and Lysak, M. A. (2011). Break-induced replication should consider chromosome structural constraints. Trends in Genet. (in press). Schubert, I., and Meister, A. (1977). Cell-cycle investigations in root-tip meristems of reconstructed karyotypes from Vicia faba. Biol. Zent. Bl. 96, 183–201. Zhang, F., Carvalho, C. M., and Lupski, J. R. (2009). Complex human chromosomal and genomic rearrangements. Trends Genet. 25, 298–307. Zhang, P., Li, W., Friebe, B., and Gill, B. S. (2008). The origin of a “zebra” chromosome in wheat suggests non-homologous recombination as a novel mechanism for new chromosome origin and step changes in chromosome number. Genetics 179, 1169–1177. Zink, D., Cremer, T., Safrich, R., Fischer, R., Trendelenburg, M. F., Ansorge, W., and Stelzer, E. H. (1998). Structure and dynamics of human interphase chromosome territories in vivo. Hum. Genet. 102, 241–251.

ACKNOWLEDGMENT

We thank Andrea Kunze and Joachim Bruder for excellent technical assistance, Stefan Heckmann for initial help with the click assay and Holger Puchta and Tim Sharbel for critical reading the manuscript.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 January 2011; paper pending published: 17 March 2011; accepted: 23 March 2011; published online: 18 April 2011.

Citation: Schubert I, Schubert V and Fuchs J (2011) No evidence for “break-induced replication” in a higher plant – but break-induced conversion may occur. Front. Plant Sci. 2:8. doi: 10.3389/fpls.2011.00008

This article was submitted to Frontiers in Plant Genetics and Genomics, a specialty of Frontiers in Plant Science.

Copyright © 2011 Schubert, Schubert and Fuchs. This is an open-access article subject to a non-exclusive license between the authors and Frontiers Media SA, which permits use, distribution and reproduction in other forums, provided that the original authors and source are credited and other Frontiers conditions are complied with.