Chromosomal rearrangements in *Arabidopsis* mutants revealed by repeated FISH

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ABSTRACT

The stability of plant nuclear genome is a necessary condition for the faithful transmission of genetic information through cell lineages. When DNA damage occurs due to various impairments, cells start a number of repair processes including ligation of broken chromosomes. As a result, dicentric chromosomes can be formed. Dicentrics are easily detectable as anaphase bridges during following mitosis. Using *Arabidopsis* as a model plant, we developed a sensitive cytogenetic assay to identify specific chromosomal rearrangements. Here we show *Arabidopsis* $tert^{-/-}$ and $atm^{-/-}$ mutants and their chromosome rearrangements and fusions analysed by fluorescence *in situ* hybridization (FISH). The method is based on successive rounds of FISH with chromosome-specific probes and the comparison of resulting FISH images.

Keywords: *Arabidopsis*; FISH; chromosomal fusions; BAC probes; rDNA loci

Although *Arabidopsis* has not been long considered a model for cytogenetic studies, the advantage of this model plant species in cytogenetics is the under-representation of interspersed repetitive DNA sequences. This allows utilization of bacterial artificial chromosomes (BACs) derived from the *Arabidopsis* sequencing project as fluorescence *in situ* hybridization (FISH) probes for physical mapping of most genomic sequences. *Arabidopsis* cytogenetics has been developed only recently (see Koornneef et al. 2003 for a review). In *Arabidopsis* deficient in major repair complexes and telomere maintenance genes (*ATM*, *TERT*), the damage of DNA can be repaired by a plethora of repair pathways; some of them result in the formation of fused chromosomes. Chromosome fusions range from simple translocations to the formation of dicentric chromosomes. Dicentrics are easily recognizable as anaphase bridges during mitotic division. In our previous studies, we performed cytogenetic characterization of the fusion events in the telomerase deficient plants and *in vitro* culture cells using rDNA and BAC-derived probes (Siroky et al. 2003, Watson et al. 2005). Functional telomeres are inevitable for the protection of eukaryotic chromosome ends. Chromosomes deprived from functional telomeres are prone to fuse at the naked termini. Telomere dysfunction was characterized in *Arabidopsis* plants harbouring a T-DNA insertion in the telomerase (*TERT*) gene (Fitzgerald et al. 1999).

Here we describe bicolour FISH assay for the identification of *Arabidopsis* chromosomes fused at either termini or intercalary sites. As probes, we use whole subtelomeric BACs obtained from Arabidopsis Stock Centres. In total, ten probes labelled with two different fluorochromes (SpectrumGreen and Cy3) are hybridized in different combinations to anaphase chromosomes in three sequential FISH. By comparing microscope images from all hybridizations, we can unambiguously identify individual chromosome arms involved in fusions.

MATERIAL AND METHODS

Plant material and preparation of microscope slides. *Arabidopsis thaliana* Columbia plants homozygous for the *tert* mutation were used

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(Fitzgerald et al. 1999). Whole terminal inflorescences were harvested and fixed in the Farmer's solution and later washed with water and transferred to 10mM citrate buffer (pH 4.5). Pistils were excised from floral buds under dissection microscope and macerated using a mixture of 0.5% Onozuka cellulase (Serva) and 0.5% Pectolyase (Sigma) at 37°C in a moist chamber for 3 h. After washing in citrate buffer pistils were transferred into a drop of 60% acetic acid on microscope slides and squashed under cover slip. The cover slip was removed by dry-ice technique and slides were post-fixed in the Farmer's solution and air-dried.

FISH probes. Probes for individual termini of *Arabidopsis* chromosomes were selected using the Arabidopsis Information Resource (TAIR, Rhee et al. 2003). Individual BAC clones were selected so as they do not contain long tracts of repetitive sequences, and positioned either directly adjacent to telomeres or at closest available positions. BAC DNA was isolated from 500 ml of bacterial cultures using QIAGEN Plasmid Midi Kit. As a probe for labelling of the short arms of chromosomes 2 and 4, an internal 2478 bp (*Eco*R I) fragment of the 25S-rRNA gene was used (Kiss et al. 1989). For bicolour FISH the probes were labelled either with the SpectrumGreen-dUTP (Vysis) or Cy3-dUTP (Amersham) by nick translation.

***In situ* hybridization and acquisition of images.** Mitotic preparations were digested with RNase A (Qiagen, 100 µg/ml in 2 × SSC, 1 h at 37°C) and pepsin (Sigma, 50 µg/ml in 0.01N HCl, 10 min, RT). After post-fixation in 3.7% neutral formaldehyde (10 min) and washing in 2 × SSC, the slides were dehydrated in an increasing ethanol series and air-dried. The hybridization mix (30 µl per slide) contained 60 ng of each labelled BAC DNA and/or 20 ng of labelled rDNA probe, 10% dextran sulphate, and 50% formamide in 2 × SSC. Heat-denatured hybridization mixture was applied on slides, covered with plastic cover slips, and the slides subjected to heat denaturation and gradual lowering of the temperature to 37°C. After hybridization at 37°C for 18 h, slides were washed and stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). Slides were visualized using the Olympus AX 70 fluorescent microscope. Images were registered by a CCD camera attached and processed with the ISIS imaging software (MetaSystems). Coordinates of each particular field of view were automatically recorded using motorized microscope stage (Maerzhaeuser). For the second and third FISH, cover slips were removed and the slides were washed twice with 2 × SSC. After re-fixation in the Farmer's fixative the slides were subjected to hybridization procedure starting with the denaturation step. During the denaturation, the probes previously hybridized to targets were washed out.

RESULTS AND DISCUSSION

The probes used for tagging of the specific chromosomes are depicted in Table 1. The discrimination between 2S and 4S chromosome ends (both with rDNA sites) was enabled using alteration of 4S-specific F6N15 BAC probe in the first FISH and 25S rDNA probe in the third hybridization instead of the single 25S rDNA probe for the chromosome 2. The resulting patterns of three respective FISH procedures are schematically illustrated in Figure 1. In the first FISH experiment six probes were used: three labelled with the SpectrumGreen (white rectangles) and three with Cy3 (grey rectangles). In the second FISH, two red and two green probes were used, followed by the use of one red and two green probes in the third hybridization.

Using the described experimental setup, we were able to show that chromosomes in the telomerase deficient plants (*tert*$^{-/-}$) with ablated telomeres fuse preferentially at the very distal

Table 1. Probes used in tagging of *Arabidopsis* chromosomes

Chromosome arm*	Annotation unit	GenBank Accession No.
1S	F6F3	AC023628
1L	F5I6	AC018848
2S	(25S rDNA)**	X13557
2L	F11L15	AC007927
3S	F16M2	AL138648
3L	T4P13	AC008261
4S	F6N15	AF069299
4S	(25S rDNA)**	X13557
4L	T5J17	AL035708
5S	F7J8	AL137189
5L	K9I9	AB013390

Terminal BAC clones are shown with assignment to chromosomes; *S and L letters refer to Short and Long chromosome arms; rDNA probe (**) is included with GenBank accession number

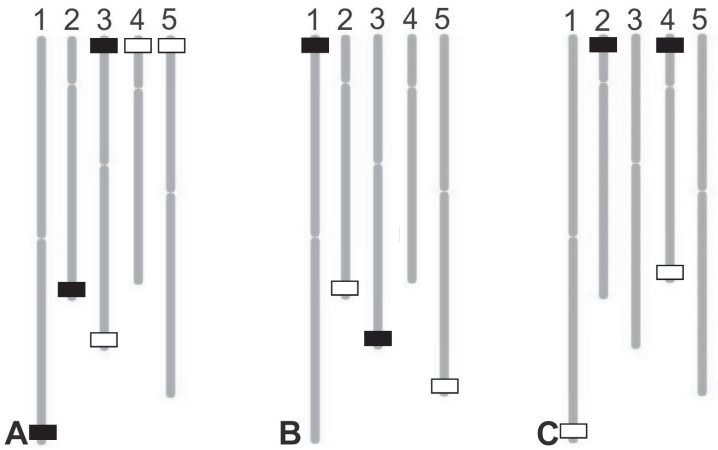

Figure 1. Scheme of consecutive FISH experiments

In three sets of FISH, the following terminal chromosome-specific probes were used: (A) The first hybridization: Three labelled red (for ends of chromosomes 1L, 2L and 3S) and three labelled green (for ends of chromosomes 3L, 4S and 5S). (B) The second hybridization: Red probes for 1S and 3L, and green probes for 2L and 5L. (C) The third hybridization: Red probe for 2S and 4S and green probes for 1L and 4L. In the 3rd FISH the red labelled probes are rDNAs (C). All other probes used in the described experimental setup are BAC-derived. *Arabidopsis* chromosomes carry NORs at the 2S and 4S positions of chromosomes. The use of BAC-probe F6N15 labelled with SpectrumGreen-dUTP (green colour) in the 1st FISH enabled distinguishing NORs from chromosome 2 and 4.

ends. The absence of protective complexes at the ends of chromosomes of *tert*$^{-/-}$ mutants makes the chromosome termini accessible to exonucleases, which contribute to further telomere shortening and/or ablation. Uncapped chromosome ends can be recognized by cell internal repair factors as double strand breaks (Ferreira et al. 2004). In telomerase null plants from early generations (5th to 6th generations) the onset of chromosome fusions typically formed by joining the chromo-

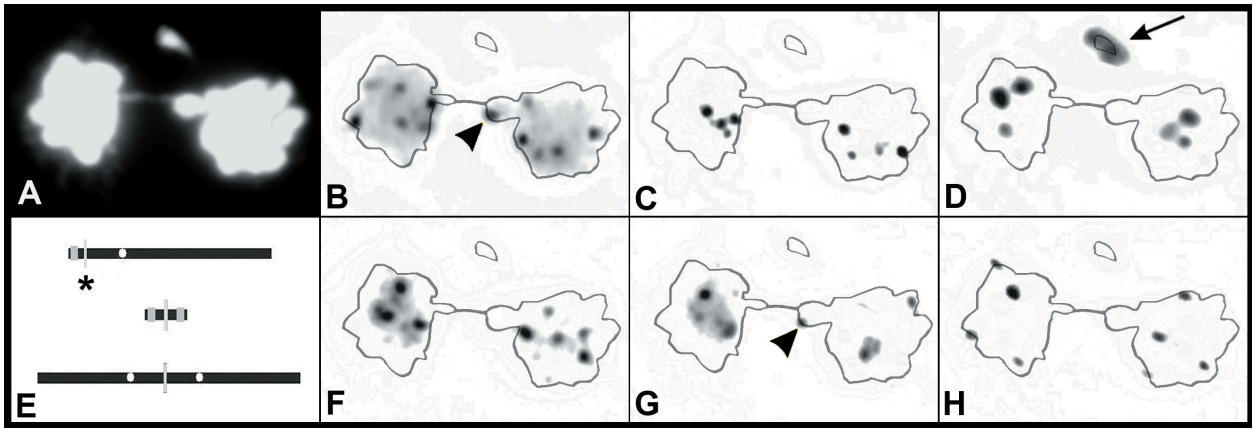

Figure 2. Chromosomal rearrangement in the *atm* mutant

The breakage with consequent intercalary fusion of chromosome 2. (A) DAPI image of anaphase displaying the anaphase bridge. (B, C, D and F, G, H) inverted black and white images of FISH. (B, C, D) red signals in consecutive 1st, 2nd and 3rd FISH, (F, G, H) green signals in consecutive 1st, 2nd and 3rd FISH. Chromosome 2 can be identified by the occurrence of 2L-specific red signal in (B, arrowhead) alternating with green signal in (G, arrowhead). Acentric chromosomal fragment bearing two rDNA signals is visible in (D), arrow. (E) Schematic representation of chromosome 2 rupture (asterisk) and consequent fusions.

somes end-to-end was found (Riha et al. 2001). Late generation of *tert*$^{-/-}$ mutants (8th generation) exhibited massive chromosome rearrangements involving fusions of chromosomes at intercalary sites (Siroky et al. 2003).

Similarly, the mutant plants deficient in repair genes display a great spectrum of chromosome fusions as a consequence of DNA breaks. In *Arabidopsis* plants deficient in repair and checkpoint protein ATM, the randomly introduced DNA breaks are repaired either by homologous recombination or by non-homologous end-joining mechanisms. The later case can be easily detected and proved by this cytogenetic technique (Figure 2). This particular anaphase bridge in *atm*$^{-/-}$ plant can be interpreted as an outcome of breakage in short arm of chromosome 2 at intercalary position.

The identification of chromosomes in cytological preparations by means of BAC-FISH painting technique, originally developed for *Arabidopsis* (Lysak et al. 2001), could also be used to precisely follow the individual chromosomes and/or chromosome arms. In comparison with painting techniques the method presented here is less laborious, relatively cheap and can be used routinely to inspect chromosome fusion events utilizing BAC clones as specific chromosome "tags".

The identification of respective chromosome ends in *Arabidopsis* mutants using repeated FISH with BAC-derived probes represents a robust method in describing chromosome fusion events. The collections of BAC clones of *A. thaliana* are accessible to scientific public and extensive data are available on the Internet (Rhee et al. 2003). Due to a relatively small size of *Arabidopsis* genome and its low content of repetitive DNA, a majority of BAC-clones give only negligible non-specific FISH signals. During the evaluation of slides, individual images of any particular field of view in the microscope are to be found repeatedly. The use of motorized microscope stage with automatic co-ordinates storage is not necessary, but appropriate.

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