

Functional analysis of the *Arabidopsis* centromere by T-DNA insertion-induced centromere breakage

Minoru Murata*[†], Etsuko Yokota*, Fukashi Shibata*[‡], and Kazunari Kashiwara*

*Research Institute for Bioresources, Okayama University, Kurashiki 710-0046, Japan; and [‡]Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

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Two minichromosomes (α and δ) in addition to two other aberrant chromosomes (β and γ) were found in a transgenic *Arabidopsis* plant produced by an *in planta* vacuum infiltration technique. The minichromosomes were successfully separated by successive crossing and selfing and added to wild-type Columbia (Col-0) as a supernumerary chromosome. FISH indicated that both of the two minichromosomes originated from the short arm of chromosome 2. The mini α chromosome contained the whole short-arm 2S and a truncated centromere (180-bp repeat cluster), whereas mini δ lacked the terminal region including telomere repeats. Pachytene FISH clearly revealed that mini δ comprised a ring chromosome carrying two copies of the region from the 180-bp repeat cluster to BAC-F3C11. Both of the 180-bp clusters (each \approx 500 kb in length) were thought to possess normal centromere functions because the centromere-specific histone H3 variant (HTR12) was detected on both clusters. Notwithstanding this dicentric and ring form, mini δ was stably transmitted to the next generations, perhaps because of its compact size (<4 Mb). Chromosome β also comprised a dicentric-like structure, with one of the two 180-bp repeat sites derived from chromosome 1 and the other from chromosome 2. However, the latter was quite small and failed to bind HTR12. The data obtained in this study indicated that 500 kb of the 180-bp array of the chromosome 2 centromere, from the edge of the 180-bp array on the short-arm side, is sufficient to form a functional domain.

minichromosomes | ring chromosome

The centromere is a multifunctional complex in eukaryotic chromosomes that is involved with the accurate segregation of chromatids to opposite poles during mitosis and with the segregation of half-bivalents and chromatids at the first and second divisions of meiosis, respectively. This function also involves kinetochore formation, microtubule attachment, chromosome movement, heterochromatin establishment, and mitotic checkpoint control. In many higher eukaryotes, tandemly repetitive DNA sequences have been found as a main component of the centromere (1, 2). In *Arabidopsis thaliana*, these repeats are comprised of a 180-bp repeat family, with units mainly of 178 bp in length (3–5). The cluster size of the 180-bp cores has been estimated to be 2.7–3 Mb in all five chromosomes (6). This size of the repeat was once thought to be critical in maintaining the centromeres, but proteins such as HTR12 (*Arabidopsis* CENP-A homologue), AtCENP-C, and AtMIS12 have been shown to assemble in only limited parts of the 180-bp repeat clusters (7–10). This indicated that short arrays of centromeric DNA repeats are sufficient to bind kinetochore proteins.

To identify the minimum functional unit that confers centromere function, minichromosomes carrying truncated centromeres have been investigated in *Drosophila* (11), humans (12), and maize (13). Recently, we found a minichromosome in *A. thaliana* derived from the short arm of chromosome 4 (14). The size of this “mini 4S” chromosome and the amount of centromeric major satellite (180-bp family) were determined to be \approx 7.5 and 1 Mb, respectively. The initial aim of the present study was to introduce marker genes into this mini 4S for subsequent

manipulation. Rather than producing T-DNA-tagged mini 4S chromosome, other minichromosomes were produced after T-DNA insertion in the centromeric region of chromosome 2. Here, we describe two types of minichromosomes (α and δ) as well as other aberrant ones (β and γ). Chromosomes δ and β display special features in that the former is a stable dicentric ring minichromosome, whereas the latter is a pseudodicentric. This phenomenon of T-DNA insertion-induced centromere breakage may provide a powerful tool for manipulating plant chromosomes and facilitating the analysis of the functional structures of centromeres.

Results

Chromosomal Aberrations Induced by T-DNA Insertions. Approximately 50 transformants (T_1) were produced *in planta* and then subjected to FISH analysis by using pBGF101 [supporting information (SI) Fig. S1] and a 180-bp repeat DNA as probes. Among these, one transformant, referred to as G40, was found to contain a T-DNA-tagged minichromosome (Fig. 1A). Subsequent FISH using 18S and 5S rDNA probes revealed that four different types of aberrant chromosomes were involved in this plant, which possessed a total of 13 chromosomes. These aberrant chromosomes were named α , β , γ , and δ (α , minichromosome with T-DNA insertion; β , large chromosome with T-DNA; γ , a small chromosome with T-DNA; δ , the smallest minichromosome without T-DNA but with short 180-bp clusters) (Fig. 1B). Because the plants carrying a pair of mini 4S (14) were used for transformation, a mini 4S chromosome also appeared in the transformant. An additional T-DNA insertion was found in chromosome 5 (Fig. 1A and B).

The transformant plant G40 was fertile, and after selfing, T_2 seeds were collected and germinated for further analyses. The plants obtained (G40-1, -2 etc.) were crossed with ecotype Columbia (Col-0) to investigate the T-DNA insertion and chromosome conformation.

Structure and Origin of Two Minichromosomes. At the back-cross generation, $B_1 = \text{Col-0}(\text{♀}) \times T_2(\text{♂})$, two types of minichromosomes, with and without T-DNA insertion, referred to as “ α ” and “ δ ,” respectively, were separated from the other aberrant chromosomes, and each was added to wild-type Col-0 as a supernumerary in the subsequent generations. The minichromosomes were considerably smaller than the normal chromosomes and hence easily distinguishable. FISH confirmed that α carried 18S but no 5S rDNA (Fig. 1C and D), suggesting that it had originated from the short arm of chromosome 2 (2S) because the other 18S rDNA cluster on the short arms of chromosome 4 is

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The authors declare no conflict of interest.

[†]To whom correspondence should be addressed: E-mail: mmura@rib.okayama-u.ac.jp.

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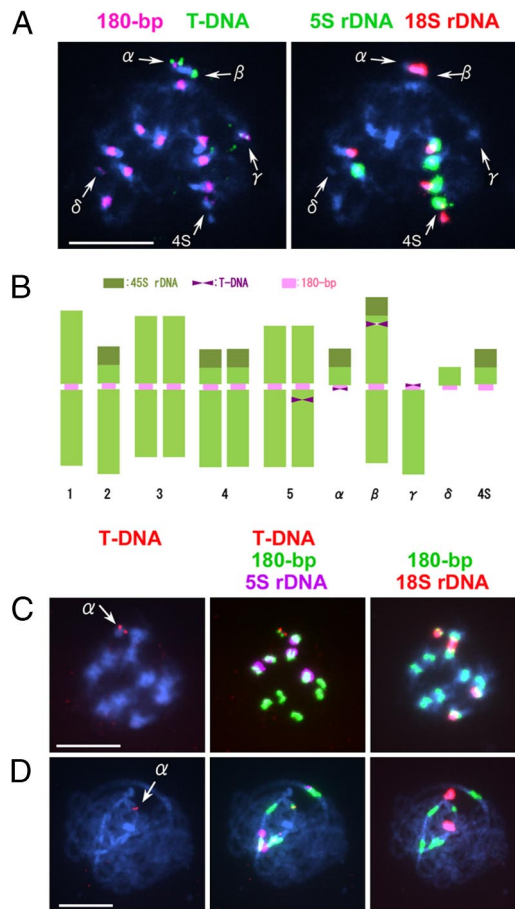


Fig. 1. Cytological analysis of a transgenic plant, G40 (T_1 generation) and its offspring carrying minichromosome α . (A) A metaphase cell of G40 with four different aberrant chromosomes (α , β , γ , and δ) in addition to mini 4S. (B) Schematic representation of chromosome constitution of G40 estimated from A and other FISH images. (C) Mitotic metaphase. (D) Pachytene nucleus of a Tr α plant with a mini α (arrow) as a supernumerary chromosome. The centromere-specific 180-bp repeat, 5S and 18S rDNA, and pBG101 DNA to detect T-DNA insertion sites were used as probes. (Scale bars: 5 μm .)

associated with 5S rDNA (5, 15). FISH using all BAC clones from the short arm of chromosome 2 yielded signals on chromosome α (data not shown), thus confirming the proposed origin. The minichromosome was designated as “mini α ,” and the line carrying it was designated as “Tr α .” However, it was later found that the terminal tip from the top arm of chromosome 1 is attached to the centromeric region of mini α . This indicates that the rearrangement involves 2S and the top arm of chromosome 1. Compared with the wild-type (Col-0), no distinct phenotypic changes appeared in these plants except the presence of kanamycin-resistance and green fluorescence in the root tips. These two features are due to expression of T-DNA.

Mini δ also segregated as an additional chromosome to the normal set of 10 chromosomes ($2n = 10 + \delta$) (Fig. 2A). It was smaller than mini α , and no T-DNA insertion was detected in it. BAC clones derived from the pericentric region of chromosome 2S, but not the terminal BAC clones nor 18S rDNA, yielded FISH signals on mini δ . Thus, mini δ also originated from the short arm of chromosome 2 but lacks the terminal region. Pachytene chromosomes probed with the centromere-specific 180-bp repeats and with pericentric BAC clones revealed 180-bp repeats at both ends of mini δ , each associated with a FISH signal of the BAC clone F3L12 (Fig. 2B), suggesting a circular structure for mini δ . Indeed, some pachytene cells revealed a distinct ring

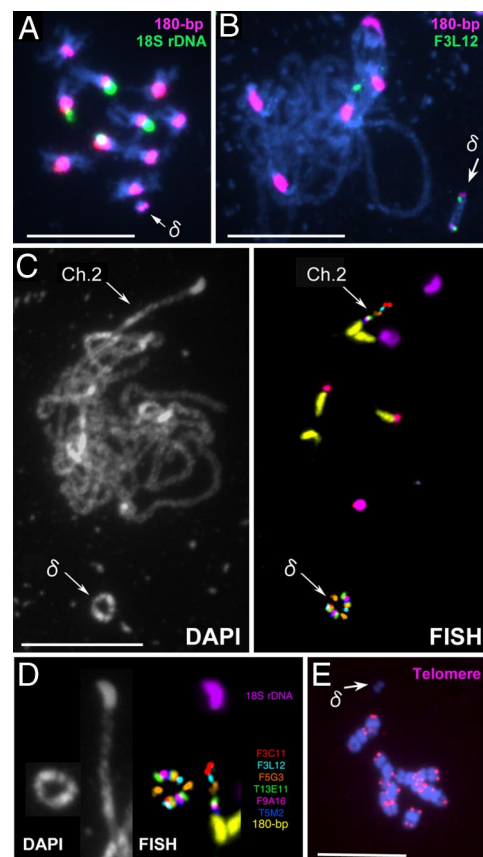


Fig. 2. FISH images of Tr δ nuclei with mini δ (arrow) as a supernumerary chromosome. (A) A mitotic metaphase cell probed with 180-bp repeats (pink) and 18S rDNA (green). (B) Pachytene chromosomes probed with 180-bp repeats (pink) and F3L12-BAC (green). (C) DAPI and FISH images of pachytene chromosomes probed with 180-bp repeats (yellow), 18S rDNA (purple), 5S rDNA (red), and six different BAC clones; F3C11 (light red, AGI-map position on chromosome 2:1190978–1294851), F3L12 (light blue, 1288088–1404236), F5G3 (orange, 1829395–1953720), T13E11 (green, 3006966–3102924), F9A16 (deep red, 3112063–3214311), and T5M2 (deep blue, 3214009–3308310). (D) Side-by-side comparison of chromosomes 2 and mini δ ; the images were derived from C. (E) A mitotic metaphase cell probed with PCR-amplified telomere DNA (5'-TTTAGGG-3') (pink). (Scale bars: 5 μm .)

shape for this chromosome (Fig. 2C and D). FISH using the 180-bp repeats and six BAC clones clearly demonstrated that this ring chromosome is dicentric and comprises a head-to-tail duplication, which encloses a region from the 180-bp repeats of CEN2 up to the F3C11 sequence. The weaker signal for F3C11 on mini δ compared with that of wild-type chromosome 2 suggests a truncation of the F3C11 sequence that became fused to the 180-bp repeats, forming a ring that eventually doubled in size after a cross-over event. As expected, no telomere sequences were detected on mini δ (Fig. 2E).

Pseudodicentric and Telocentric Chromosomes. The two aberrant chromosomes β and γ could not be separated from each other, possibly because they contain large portions of chromosomes that compensate each other. Metaphase cells of plant (G40-18-6.7.3) carrying aberrant chromosomes β , γ , and mini δ are shown in Fig. 3. Chromosome β has two 180-bp repeat sites, one of which is much shorter than the other (Fig. 3A and B). As in the original G-40 plant, T-DNA insertions were found in chromosomes β and γ (Fig. 3D). Chromosome β is longer than γ and carries 18S rDNA at one terminus (Fig. 3C). Interestingly, a gap appeared between the rDNA and the T-DNA insertion, and a 180-bp repeat FISH signal,

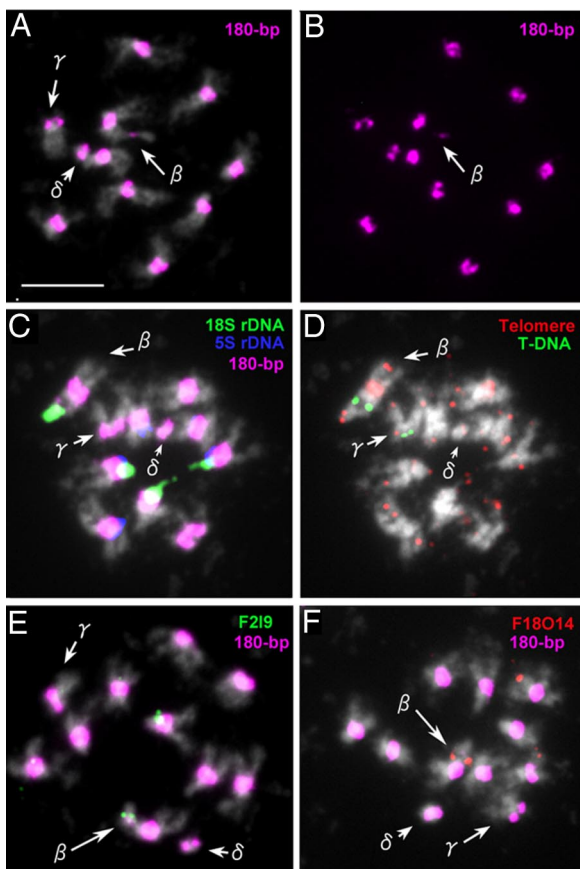


Fig. 3. Cytological analysis of a G40 offspring containing chromosomes β , γ , and mini δ . (A) A mitotic metaphase cell probed with 180-bp repeats (pink). (B) The same cell showing only 180-bp repeat signals (pink) to clearly visualize the weak signal on chromosome β . (C) A mitotic metaphase cell probed with 180-bp repeats (pink), 18S rDNA (green), and 5S rDNA (blue). (D) The same cell probed with telomeric DNA (red) and T-DNA (green). (Note: a weak telomere signal on mini δ is from an adjoining chromosome.) (E) A mitotic metaphase cell probed with 180-bp repeats (pink) and F219-BAC (green, AGI-map position of chromosome 2:173440–276564). (F) A mitotic metaphase cell probed with 180-bp repeats (pink) and F18O14-BAC (red, Altman-map of chromosome 1: 29205). (Scale bar: 2 μ m.)

although very weak, appeared to correspond to this gap (Fig. 3 C and D). This indicated that the gap region is part of the primary constriction of chromosome 2. The region distal to the gap could represent a remnant of 2S after excision of that part that became mini δ . In the region of the large 180-bp repeat cluster, a long stretch of telomeric DNA, typical for the centromeric region of chromosome 1 (16, 17), was detected (Fig. 3D). Chromosome β is most likely a pseudodicentric resulting from a translocation between chromosomes 1 and 2S, probably at their T-DNA insertion sites. This idea was confirmed by FISH with BAC clones F219 from chromosome 2S and F18O14 from the top arm of chromosome 1 (Fig. 3 E and F).

Chromosome γ , which is smaller than chromosome β , had a size that was close to the long arm of chromosome 2 (2L) (Fig. 3A). Because the other arm (2S) of chromosome 2 had been identified as mini α , it was highly probable that chromosome γ originated from the centromere breakage of chromosome 2. This assumption was confirmed by FISH with BAC clones from 2L (data not shown).

T-DNA Insertion in the 180-bp Array Coincides with the Translocation Break Point. In the original transgenic plant G40, four different T-DNA insertion sites were detected. Among these, only the

insertion in chromosome 5 was not associated with chromosomal aberrations. The other three insertion sites were either within the centromeric 180-bp repeats of mini α (2S) and γ (2L) or within β between the rDNA of chromosome 2S and the remaining top arm of chromosome 1. The insertions found on mini α and γ apparently result from a single insertion event into the centromeric region of chromosome 2.

The insertion on mini α was investigated by DNA-fiber FISH and Southern blot analyses (Fig. 4 A and B). DNA-fiber FISH clearly revealed bipartite T-DNA inserts within the 180-bp cluster, much longer than expected for a single T-DNA sequence (\approx 8 kb) (Fig. 4A). Southern blot analysis showed that three copies of T-DNA (two complete and one truncated) had probably been inserted in the centromeric satellite 180-bp repeat cluster (Fig. 4 B and C). Expression of both *GFP* and *NPTII* genes of the T-DNA was suppressed in some of the progeny, probably due to RNA interference, because two complete copies of T-DNA were inserted in a head-to-head fashion in the centromeric region.

A T-DNA insertion site on chromosome β was also detected by DNA-fiber FISH (Fig. 4A), and the insertion pattern was found to be identical with that of mini α . This suggested that chromosomes mini α and β represent derivatives of one and the same chromosome harboring an insertion.

Centromere Size and Activity in Aberrant Chromosomes. The centromeric regions of mini α , γ , and mini δ and the distal region of β were found to be truncated. Chromosomes β and mini δ possessed two 180-bp repeat clusters, and therefore, appeared as dicentric. An investigation of the repeat cluster size and centromere activity was then undertaken.

The sizes of the 180-bp repeat clusters were estimated by pulsed-field gel electrophoresis (PFGE). Because mini α and mini δ were added to the Col ecotype as supernumerary chromosomes, agarose plugs were made from lines Tr α and Tr δ . Of the 10 different restriction enzymes used, only *HpaII* could distinguish plants with and without minichromosomes (α or δ) (Fig. 5 A and B). *HpaII* cleaves 5'-CCGG-3' but not 5'-C^mCGG (CpG-methylation), and therefore, has been used to examine cytosine methylation in the centromeres of *Arabidopsis* (18, 19). Specific bands of 700 and 500 kb were detected in plants carrying mini α and mini δ , respectively. These are markedly shorter than the size reported for chromosome 2 (\approx 3 Mb) (6). The size of two 180-bp clusters on mini δ was similar because only one 500-kb band was detected.

To examine centromere activity of the 180-bp repeat clusters detected by FISH, we used an antibody against HTR12, the centromere-specific histone H3 (CENH3) of *Arabidopsis* (20), because CENH3 is a basic kinetochore protein that is bound only to functional centromeres (21). CENH3-specific immunosignals appeared on the centromeric regions of mini α and γ , as well as on all normal chromosomes (data not shown). On the ring-shaped mini δ , both 180-bp arrays revealed signals, despite their small size (\approx 500 kb) (Fig. 6A and Fig. S2A), indicating that both 180-bp sites can function as a centromere. This notion was also supported by the observation of lagging of mini δ , on the equatorial plates, when pulled from the two opposite poles (Fig. S3).

Chromosome β also revealed two 180-bp repeat sites, one of which was much smaller than the other (Fig. 3). As expected, the large 180-bp site from chromosome 1 showed a distinct HTR12 immunosignal, whereas no signals were detected on the small 180-bp repeat site (Fig. 6B and Fig. S2B). A constriction was retained at the small 180-bp-repeat site (Fig. 3A). Chromosome β was quite stable, and no anaphase bridges were observed in cells containing this chromosome. This observation indicates that chromosome β is a pseudodicentric and that only the large 180-bp site acts as a centromere.

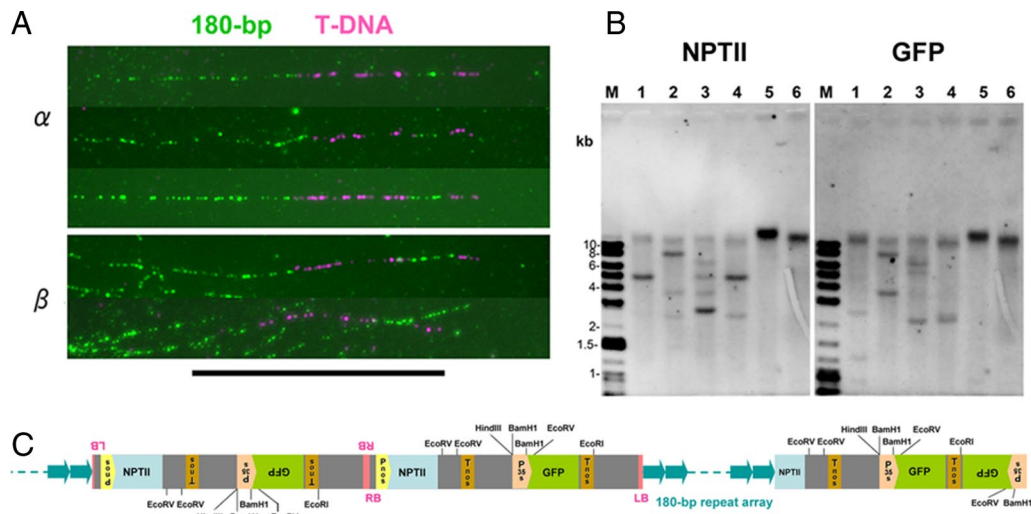


Fig. 4. DNA-fiber FISH and genomic Southern blot analyses. (A) DNA-fiber FISH with chromatin from a Tr α plant and a G40-derived plant carrying β and mini δ chromosomes, probed with T-DNA of pBGF101 (pink) and 180-bp repeats (green). (B) Southern blot analysis of genomic DNA isolated from a Tr α plant, probed with *NPTII* (neomycin phosphotransferase gene) and *GFP* (green fluorescence protein gene), M: DNA marker, lanes 1–6: BamHI, EcoRI, EcoRV, HindIII, SmaI, and XbaI digests. (C) A schematic pattern of T-DNA insertions estimated from A and B. (Scale bar: 10 μ m.)

Discussion

Based on the FISH data, we interpreted the origin of aberrant chromosomes as shown in Fig. 7A. The processes involved include the following.

1. A translocation event between the two T-DNA insertion sites in the top arm of chromosome 1 and in the centromeric 180-bp array of chromosome 2.
2. This reciprocal translocation resulted in chromosomes t11/2 and γ . The former chromosome was not maintained in the original transgenic plant G40 because it was dicentric and thus unstable. In the subsequent cell division, chromosome t11/2 was probably duplicated through nondisjunction.
3. Mini α originated from a break in the top arm region of chromosome 1 as one of the duplicated copies of t11/2 formed an anaphase bridge. Consequently, mini α contains the short arm of chromosome 2 and a small region of chromosome 1 at the opposite side of the centromeric region from chromosome 2. The larger part of t11/2, representing most of chromosome 1, was apparently lost.
4. From the other copy of t11/2, mini δ originated because of two breakages, one within F3C11 and the other within the 180-bp sequences, and subsequent joining of the internal break ends generating a ring chromosome that comprised part of the 180-bp array derived from chromosome 2. This structure was confirmed by PCR using primers from the 180-bp repeat and F3C11 sequences. The remaining two parts of t11/2, the distal

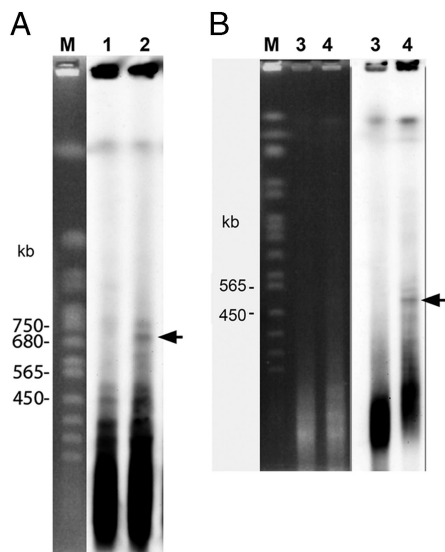


Fig. 5. PFGE/Southern blot analysis for plants carrying mini α and mini δ for size determination of the 180-bp arrays. (A) PFGE pattern of plants without (lane 1) and with mini α (lane 2). (B) PFGE pattern of plants without (lane 3) and with mini δ (lane 4); M: DNA marker from *Saccharomyces cerevisiae*. Genomic DNA was digested with HpaI. The 180-bp repeat was used as a probe. Specific bands corresponding to \approx 700 and 500 kb from plants containing mini α and mini δ , respectively, are marked with arrows.

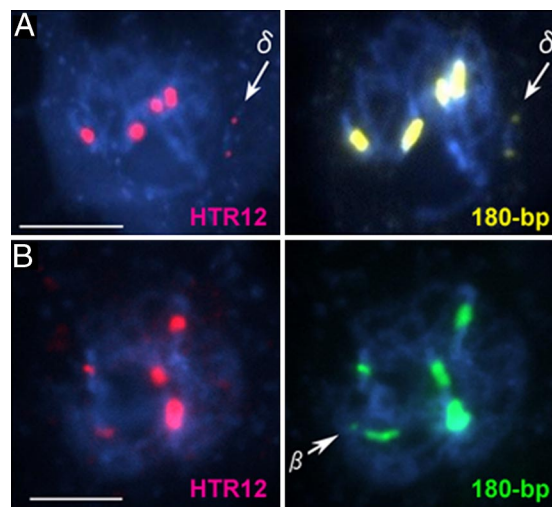


Fig. 6. HTR12-immunostaining and FISH analysis. (A) A pachytene cell of a Tr δ plant carrying mini δ after fluorescent immunolabeling by using anti-HTR12 antibodies (pink) and FISH using 180-bp repeats (yellow). The arrows indicate mini δ . (B) A pachytene cell of a plant carrying chromosome β after fluorescent immunolabeling by using anti-HTR12 antibodies (pink) and FISH using 180-bp repeats (green) to the pachytene chromosomes. The arrow indicates the small 180-bp sites on chromosome β . (Scale bars: 5 μ m.)

Although ring chromosomes have been found in both animals and plants, in general, they are not stably transmitted to the subsequent generations because of mitotic and meiotic recombination processes. The rare inheritance of human ring chromosomes has been reported (31). Mini δ is transmissible to about one-third of the selfed progeny, notwithstanding its ring and dicentric structure, and is now being maintained as a partial trisomic ($2n = 10 + \delta$). The small size (<4 Mb) could reduce recombination frequency and subsequent anaphase bridge formation. During nuclear divisions, the two 180-bp arrays behaved like a single centromere, although interlocked large-sized ring chromosomes lagging on equatorial plates appeared occasionally ($\approx 5\%$). Similarly, a ring minichromosome containing human aliphoid sequences was shown to be transmissible to the offspring through mouse spermatogenesis (32). Recently, meiotic transmission of circular centromeric DNA, which was introduced into embryonic tissues, was reported for maize, although neither circularity nor kinetochore formation has been proved (33). Nevertheless, our studies indicate that small ring-shaped chromosomes might be useful as chromosomal vectors.

Methods

Plant Material and Transformation. Three- to 4-week-old plants of the Tr45Co5 line of *A. thaliana* (Col-0) carrying a pair of mini4S chromosomes (14) were transformed by using the binary vector pBGF101 (Fig. S1) according to the *planta* vacuum infiltration method (34).

Cytological and Molecular Analyses. FISH was essentially the same as described previously (8, 14, 15) but with minor modifications. Briefly, after rinsing with milli-Q water, fixed flower buds were digested with enzyme solution [2% (wt/vol) Cellulase Onozuka RS (Yakult) and 0.5% (wt/vol) Pectolyase Y23

(Kikkoman) in citric buffer (pH 4.5)] at 37°C for 1 h. The digested tissues were fixed and dropped on cold slides. Air-dried slides were denatured in 70% formamide in $2\times$ SSC at 76°C for 1 min and then placed in 70% ethanol and stored at -20°C . For the detection of centromeres, the 180-bp repeats amplified by PCR were labeled by using the FITC High Prime kit (Roche) (8). Other *Arabidopsis* probes including BAC clones from chromosomes 1 and 2, the ≈ 500 -bp 5S rDNA repeat unit (15), and the 18S rDNA (8) were labeled either with biotin-7-dATP (GIBCO-BRL) or with digoxigenin-11-dUTP (Roche) by nick translation and detected with 5 $\mu\text{g/ml}$ (1:100 dilution) streptavidin-Cy5 conjugate (GE Healthcare) and 4 $\mu\text{g/ml}$ anti-digoxigenin Rhodamine Fab fragments (Roche), respectively. Slides were stained with 0.1 $\mu\text{g/ml}$ DAPI and examined by using a fluorescent microscope (Axioskop; Carl Zeiss). Images were captured by using a digital CCD camera (AxioCam HRm; Carl Zeiss).

For the multiprobed FISH, slides were reprobed one to three times. After detection by using the first probe, slides were placed in PBS solution to remove the coverslips, fixed again for 10 min, and then air-dried. Denaturation and hybridization were as above. The BAC clones containing unique DNA sequences were used first, followed by repetitive DNA sequences. Captured images were overlaid by using Axio Vision 3.1 (Carl Zeiss). For the indirect immunolabeling of the centromere-specific histone H3 variant (CENH3), anti-HTR12 antibodies were raised against a synthetic peptide designed from the first 20 N-terminal amino acids as reported previously (20) and applied to pachytene chromosomes prepared from pollen mother cells fixed in 4% (vol/vol) paraformaldehyde for 30 min at room temperature. Immunofluorescence signals were detected as described previously (8).

DNA fiber FISH was performed according to Fransz *et al.* (35). PFGE and genome Southern blotting were performed by using the CHEF-DRII system (Bio-Rad) as described previously (3).

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