The transposition pattern of the *Ac* element in tobacco cultured cells

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We investigated physical distances and directions of transposition of the maize transposable element *Ac* in tobacco cultured cells. We introduced a T-DNA construct that carried a non-autonomous derivative of *Ac* (designated dAc-I-RS) that included sites for cleavage by restriction endonuclease *Mlu*I. Another cleavage site was also introduced into the T-DNA region outside of the dAc-I-RS transposable element. The tobacco cultured cell line BY-2 was transformed with the T-DNA and several transformed lines that had a single copy of the T-DNA at a different chromosomal location were isolated. These lines were co-cultured with *Agrobacterium tumefaciens* cells that carried a cDNA for the *Ac* transposase gene under the control of various promoters. Sublines of cultured cells in which dAc-I-RS had been transposed, were isolated. The genomic DNAs of these sublines were isolated and digested with *Mlu*I. Sizes of DNA segments generated by digestion were determined by pulse-field gel electrophoresis. Our results showed that 20 to 70% of transposition events had occurred within several hundreds kilo-base pairs (kb) on the same chromosome. These results demonstrate that the *Ac*-*Ds* element preferentially transposed to regions near the original site in a tobacco chromosome. In addition, the present results are an example of asymmetric transposition as demonstrated by the distance of transposition on the chromosome.

INTRODUCTION

Development of recombinant DNA technology for manipulation of large segments of DNA is of importance for structural and functional analyses of plant chromosomes. The *Ac* element in maize is a transposable DNA element that moves from one site to another on chromosomes. Use of the *Ac* element has been tested for such a purpose in maize and in various heterologous plant species (Baker et al., 1986; Walbot, 1992; Bancroft et al., 1993; Chunk et al., 1993; Long et al., 1993; Jones et al., 1994; Whitham et al., 1994; James et al., 1995; Springer et al., 1995; Machida et al., 1997; Izawa et al., 1997; Enoki et al., 1999; Nakagawa et al., 2000a).

An understanding of the *Ac* element transposition pattern on a chromosome is important for its successful use in gene tagging. Previous genetic investigations have

shown that the *Ac* element is preferentially transposed to a region within the same chromosome (van Schaik et al., 1959; Greenblatt and Brink, 1962; Greenblatt, 1984; Dooner and Belachew, 1989; Jones et al., 1990; Dooner et al., 1991; Osbone et al., 1991; Moreno et al., 1992; Bancroft and Dean, 1993; Carrol et al., 1995; Machida et al., 1997; Ito et al., 1999; Nakagawa et al., 2000a). Insertion mutation of genes near the chromosomal location of the *Ac* element have, in fact, been obtained and the causative genes have been cloned (Delong et al., 1993; Jones et al., 1994; Whitham et al., 1994; James et al., 1995; Seki et al., 1999). Preferential transposition of *Drosophila P* elements to nearby chromosomal sites has also been reported (Tower et al., 1993). In Arabidopsis plants, we have shown that 50% of transposition events involving dAc-I-RS, a derivative of the *Ac* element constructed in our laboratory, had occurred within 1,700 kb on the same chromosome, with 35% within 200 kb. Although the *Ac* element has been shown to preferentially transpose to linked chromosomal locations in tobacco plants (Jones et

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al., 1990), the pattern of *Ac* transposition at the molecular level has not yet been analyzed.

In the present study, we investigated the efficiency of reinsertion after *Ac* excision, and determined distances and directions of *Ac* transposition on the chromosomes of tobacco BY-2 cultured cells. In 20 to 70% of BY-2 cell lines in which transposition occurred, this element was transposed to various locations on the chromosome within a region of approximately several hundreds kilo-base pairs (kb) from the original site. The importance of understanding the *Ac* transposition pattern is discussed with regard to the manipulation of chromosomes and gene tagging in tobacco plants.

MATERIALS AND METHODS

Plant cells and bacterial strains. Suspension cultures of the cell line BY-2 of tobacco (*Nicotiana tabacum* L. cv. Bright Yellow 2) have been described elsewhere (Kato et al., 1972). BY-2 cells were used for isolation of transgenic lines T/dAc#8, #12, #13 and #19, which carried a single copy of (I-RS/dAc-I-RS)T-DNA (Fig. 1A). *Agrobacterium tumefaciens* EHA101 (Hood et al., 1986) and LBA4404/pAL4404 (Hoekema et al., 1983) were used as the hosts for BY-2 transformation.

Enzymes, chemicals and plasmids. Restriction and modifying enzymes were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan) or Toyobo Co. Ltd. (Osaka, Japan). Hygromycin B and kanamycin sulfate were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Cellulase Onozuka RS and Pectolyase Y-23 for the preparation of protoplasts were purchased from Yakult Co. Ltd. (Tokyo, Japan) and Seisin Pharmaceutical Co. Ltd. (Tokyo, Japan), respectively.

Plasmids and their relevant characteristics are listed in Table 1. The binary plasmid pGA Δ NI-RS/dAc-I-RS,

which contained (I-RS/dAc-I-RS)T-DNA (Fig. 1A) (The GenBank accession number of dAc-I-RS is AB055064), and the binary plasmid pGAH Δ N35S Δ CTPase, which contained the cDNA that correspond to the gene for *Ac* transposase were described previously (Kunze et al., 1987; Machida et al., 1997). The binary plasmids pGAH Δ Npar Δ CTPase and pGAH Δ Np2'AcTPase contained the *Ac* transposase gene under the control of *parA* promoter (Niwa et al., 1994) and 2' promoter (Velten et al., 1984), respectively.

Induction of dAc-I-RS transposition by introducing *Ac* transposase and selection of plant cells with a transposed dAc-I-RS. A modified co-culture method was used for transient introduction of the *Ac* transposase gene into the transformed BY-2 cells. Cells of *A. tumefaciens* EHA101 harboring pGAH Δ N35S Δ CTPase, pGAH Δ Npar Δ CTPase or pGAH Δ Np2'AcTPase were cultured until the absorbance at 600 nm was 1.5, and the culture was concentrated four-fold. A 100 μ l aliquot of this culture was mixed with 4 ml of a suspension culture of transformed BY-2 cell lines, T/dAc #8, #12, #13, and #19, which carried a single copy of (I-RS/dAc-I-RS)T-DNA. The mixture was incubated in Linsmaier and Skoog (LS) medium (Linsmaier and Skoog, 1965) containing 0.2 mg/l 2,4-D at 22°C in the dark for 24–144 hr. Cells were selected for resistance to kanamycin in LS medium, which was supplemented with 0.2 mg/l 2,4-D and 100 or 200 mg/l kanamycin. Cells that showed resistance to kanamycin were transferred to a plate containing LS medium supplemented with 0.2 mg/l 2,4-D, 100 mg/l kanamycin and 50 mg/l hygromycin to check for reinsertion of dAc-I-RS.

Extraction of DNA and PCR analysis. DNA was extracted from the cells by the cetyltrimethylammonium bromide method (Murray and Thompson, 1980) and used as a template for amplification by the polymerase chain reaction (PCR). PCR was carried out in a DNA Thermal

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pGA Δ NI-RS/dAc-I-RS	Derivative of pGAH Δ N containing the (I-RS/dAc-I-RS)T-DNA	Machida <i>et al.</i> (1997) (Fig. 1) (GenBank ac. no. AB055064)
pGAH Δ Npar Δ CTPase	Derivative of pGAH Δ N containing the cDNA for <i>Ac</i> transposase gene under the control of <i>par</i> promoter	Machida <i>et al.</i> (1997)
pGAH Δ Np2'AcTPase	Derivative of pGAH Δ N containing the cDNA for <i>Ac</i> transposase gene under the control of 2' promoter	This study
pGAH Δ N35S Δ CTPase	Derivative of pGAH Δ N containing the cDNA for <i>Ac</i> transposase gene under the control of P35S	Machida <i>et al.</i> (1997)
pGA492	Binary vector plasmid containing the neomycin phosphotransferase II gene and a multiple cloning site	An (1987)
pGA492 Δ NBP	Derivative of pGA492 containing a deletion of the region of the neomycin phosphotransferase II gene	This study
pGAH Δ N	Derivative of pGAH containing a deletion of the region of the neomycin phosphotransferase II gene	Onouchi <i>et al.</i> (1995)

Cycler (Perkin-Elmer Cetus, USA) using 0.1 µg of genomic DNA in a total volume of 50 µl. The amplification included 30 cycles of 30 second denaturation at 94°C, 1 min annealing at 60°C and 3 min elongation at 72°C. The reaction mixture was analyzed on a 4% polyacrylamide gel. The sequences of the primers (Fig. 1A) were 5'-TAC-TTACGTCACGTCTTGCG-3' (1'pro), 5'-ACCTGCGTGC-AATCCATCTT-3' (NPTIUP), 5'-TTACCTCGGGTTCGA-AATCG-3' (AcUP), 5'-GGATGTATGGCAGCATTTC-3' (ACL1), and 5'-GTCCACAGTGATATGATCTC-3' (RS1).

Preparation of high molecular weight DNA and digestion with *Mlu*I. High molecular weight DNA from tobacco BY-2 cultured cells was isolated from protoplasts prepared by the method of Nagata *et al.* (1981). Protoplasts were suspended in 0.6 M mannitol, at a final concentration of approximately 2×10^8 protoplasts/ml. High molecular weight DNA was prepared from protoplasts following the methods of Guzmán and Ecker (1988) and Machida *et al.* (1997). For digestion with *Mlu*I, agarose plugs were placed in a solution of 50 mM EDTA (pH 8.0) and incubated for 2 hr at 25°C to inactivate proteinase K. The plugs were then transferred to a solution of 50 mM EDTA (pH 8.0) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated overnight at 4°C. The plugs were next transferred to a solution containing 10 mM Tris-HCl and 0.1 mM EDTA (pH 8.0) and incubated for 2 hr at 25°C. After transfer to fresh solution, the plugs were stored at 4°C until use. The plugs were transferred to *Mlu*I reaction solution and incubated for 2 hr at 37°C, twice, and then transferred to fresh solution and incubated for 16 hr at 37°C after the addition of *Mlu*I.

Conditions for pulse-field gel electrophoresis. Pulse-field gel electrophoresis (PFGE) was performed as previously described (Machida *et al.*, 1997) in an LKB pulsaphore (PharmaciaLKB, Uppsala, Sweden).

RESULTS AND DISCUSSION

Experimental design for measurement of transposition distances by *Mlu*I. Fig. 1A shows the T-DNA construct we made for the investigation of distances and direction of transposition. The T-DNA construct contained the *Mlu*I recognition site inside and outside of the modified *Ac* transposable element [we designated this element and the T-DNA, dAc-I-RS and I-RS(dAc-I-RS)T-DNA, respectively (Machida *et al.* 1997)]. When transposition of dAc-I-RS occurs within the same chromosome, digestion of genomic DNA by *Mlu*I should give rise to a segment of chromosomal DNA flanked by part of the T-DNA at the original integration site and part of the transposed dAc-I-RS sequence (see Fig. 1B). Using two DNA fragments as probes (probes 1 and 2) for Southern hybridization, we were able to distinguish the relative direction

Fig. 1. (A) Schematic diagram of the (I-RS/dAc-I-RS)T-DNA construct in plasmid pGAΔNI-RS/dAc-I-RS. dAc-I-RS indicates the non-autonomous derivative of the *Ac* element that contained the cleavage site for restriction endonuclease *Mlu*I and *RS* sequences (see text). RB and LB indicate right and left border sequences on the T-DNA, respectively. 1' promoter, the 1' promoter of the octopine Ti plasmid TR-DNA; P35S, the promoter of the gene for 35S RNA from cauliflower mosaic virus; HPT, the coding sequence of the gene for hygromycin phosphotransferase; NPTII, the coding sequence of the gene for neomycin phosphotransferase II; Tnos, the terminator of the gene for nopaline synthase; Tocs, the transcriptional terminator of the gene for octopine synthase; IR, the terminal inverted repeat sequences of *Ac*; *RS*, the recombination site that is recognized by the R protein from *Zygosaccharomyces rouxii*. Thick lines indicate the probes (probes 1 and 2) used for Southern blotting analysis. Arrowheads indicate the primers used for PCR (Materials and Methods). (B) The strategy for measurement of the distance of transposition. *Mlu*I indicates sites of cleavage by the restriction endonuclease *Mlu*I. Putative cleavage sites for *Mlu*I are also indicated on plant DNAs. A dark gray box indicates dAc-I-RS. Light gray boxes indicate regions of T-DNA other than dAc-I-RS in (I-RS/dAc-I-RS)T-DNA.

of each transposition. We also introduced *RS* sequences, namely, recognition sites for recombinase (R protein) from *Zygosaccaromyces rouxii* (Araki et al., 1985, Matsuzaki et al., 1988, Araki et al., 1992) into the T-DNA construct: two *RS*s in inverted orientation were placed inside and outside dAc-I-RS, respectively, as described previously (Machida et al., 2000). In addition to such specific DNA sequences, we inserted the gene for hygromycin phosphotransferase (HPT) under the control of a 35S promoter into the dAc-I-RS element for selection of transgenic cell lines and detection of dAc-I-RS insertion (Fig. 1B). For monitoring the excision of dAc-I-RS, this element was inserted between the 1' promoter (Velten et al., 1984) and the coding sequence of the element for neomycin (kanamycin) phosphotransferase II (cryptic NPTII) (Baker et al., 1987). Thus, excision of dAc-I-RS should create an active NPTII gene in transgenic Arabidopsis plants that contain (I-RS/dAc-I-RS)T-DNA.

Isolation of transgenic tobacco cultured BY-2 cell lines that contained the (I-RS/dAc-I-RS)T-DNA and induction of transposition. We isolated transformed tobacco cultured BY-2 cells that contained (I-RS/dAc-I-RS)T-DNA, and we selected four transgenic lines that had a single copy of the T-DNA. We designated these lines T/dAc#8, #12, #13 and #19.

In order to express the *Ac* transposase, we linked three types of promoter sequences to the cDNA encoding the transposase: the promoter of the tobacco *par* gene, whose activity was shown to be higher in BY-2 cells than that of

the 35S promoter of cauliflower mosaic virus (P35S) (Niwa et al., 1994), P35S (Guilley et al., 1982), and the promoter of the 2' gene from T-DNA (Velten et al., 1984). We co-cultured the transformed BY-2 lines with cells of *A. tumefaciens* EHA101 harboring pGAHΔN Δ parAcTPase that carried the gene for *Ac* transposase under the control of the *par* promoter (Materials and Methods). Numbers of kanamycin resistance cells were counted at various times after an onset of co-culturing. As shown in Table 2, kanamycin resistance cells appeared in both T/dAc#12 and #19 lines 48 hours after co-culturing and numbers of kanamycin resistance calli increased as co-culturing continued (Table 2, lanes 1 and 3). When cells were co-cultured with *Agrobacterium* that harbored pGA492-ΔNBP, which did not contain the *Ac* transposase gene, no resistant callus was observed (Table 2, lanes 2 and 4). These results show that our construct encoding *Ac* transposase can mediate excision of dAc-I-RS in tobacco cultured cells. We also examined the frequency of excision mediated by the *Ac* transposase gene under the control of promoters other than the *par* promoter. As shown in Table 3, P35S was shown the most effective for introducing excision. We decided to use the construct pGAHΔN35S Δ AcTPase for induction of transposition in further experiments.

To examine DNA structures after induction of dAc-I-RS excision in each transformed cell line, we established sub-lines of cells that showed resistance for kanamycin after co-culturing with the *Agrobacterium* cells containing the *Ac* transposase gene. Total chromosomal DNAs were

Table 2. Numbers of Km^r calli after onset of coculture with *Agrobacterium* cells that carried the *Ac* transposase gene.

Cryptic NPTII gene in transformed line	Binary vector	Number of Km ^r calli of BY-2 transformed cells with the cryptic NPTII after coculture with <i>Agrobacterium</i> cells that carried the <i>Ac</i> transposase gene for			
		24 hr	48 hr	72 hr	96 hr
T/dAc#12	pGAHΔN Δ parAcTPase	0	9	26	126
T/dAc#12	pGA492ΔNBP	0	0	0	0
T/dAc#19	pGAHΔN Δ parAcTPase	0	6	17	59
T/dAc#19	pGA492ΔNBP	0	0	0	0

7 × 10⁵ cells of BY-2 were examined on each experiment.

Table 3. Numbers of Km^r calli after onset of coculture with *Agrobacterium* cells that carried the *Ac* transposase gene under the control of various promoters.

Cryptic NPTII gene in transformed line	Binary vector	Promoter for transposase gene	Number of Km ^r calli
T/dAc#12	pGAHΔN Δ parAcTPase	<i>par</i> promoter	30
T/dAc#12	pGAHΔNp2'AcTPase	2' promoter	1
T/dAc#12	pGAHΔN35S Δ AcTPase	P35S	43
T/dAc#12	pGA492ΔNBP	no	0
T/dAc#19	pGAHΔN Δ parAcTPase	<i>par</i> promoter	22
T/dAc#19	pGAHΔNp2'AcTPase	2' promoter	0
T/dAc#19	pGAHΔN35S Δ AcTPase	P35S	23
T/dAc#19	pGA492ΔNBP	no	0

7 × 10⁵ cells of BY-2 were examined on each experiment.

purified from the kanamycin resistant cells and subjected to analysis by PCR using appropriate combinations of oligonucleotide primers (Fig. 1). We used primers 1'pro and NPTIIUP for detection of empty sites after excision of the dAc-I-RS element. As shown in Fig. 2, when the cells of the T/dAc#8-K2 and K3 sublines that showed resistance to kanamycin were examined, approximately 261-bp fragments were amplified (lane 1). Polynucleotides of this length are expected if excision of the dAc-I-RS occurs. However, when the primers 1'pro and AcUP, by which we could examine the preexcision sequence, were used, no fragment was amplified in these lines (lane 2). When the genomic DNA of the cells in the T/dAc#8 line (before induction of transposition) was examined using 1'promoter AcUP primers, a 277-bp fragment was amplified (Fig. 2, lane 2).

When another combination of primers (AcL1 and RS1, by which we could examine the presence of dAc-I-RS) was used, DNA fragments with expected sizes were amplified before and after induction of transposition (Fig. 2, lane 3). These data suggest that dAc-I-RS was reinserted into chromosomal DNA in each of the cell lines.

The sublines of cells that showed resistance to kanamycin were examined for resistance to hygromycin. We also

carried out Southern blotting and PCR analysis using genomic DNAs from the cells to examine the reinsertion of dAc-I-RS. Between 33 and 88% of the sublines that exhibited resistance to kanamycin were resistant to hygromycin and carried transposed dAc-I-RS elements (Table 4), but they did not have a stable *Ac* transposase gene.

Measurement of physical distances of transposition.

High-molecular-weight chromosomal DNA from the T/dAc#8 line before transposition was purified, digested with *Mlu*I, and DNA segments were fractionated by pulse-field gel electrophoresis (PFGE) and subjected to Southern blot analysis using probes 1 and 2 (see Fig. 1). When probe 2 was used, the 700-kb fragment was detected as shown in Fig. 3 (lane 1). With probe 1, the 400-kb fragment was similarly detected (data not shown). Thus, in the genomic DNA of the T/dAc#8 cell line, the *Mlu*I sites nearest to the (I-RS/dAc-I-RS)T-DNA insertion site were mapped 400 kb and 700 kb toward RB and LB, respectively (see Fig. 4). When dAc-I-RS transposes within these *Mlu*I sites in the T/dAc#8 line, the distances and directions of transposition can be measured by *Mlu*I.

Subsequently, we analyzed *Mlu*I digests of chromosomal DNAs from eight sublines of cells, which were derived from the T/dAc#8 line and had transposed dAc-I-RS elements. In five out of eight sublines, detected DNA bands were shorter in size than the detection limits described above. Fig. 3 shows typical results of such an analysis: in four sublines, DNA fragments of 5 kb, 550 kb, and 200 kb were detected with probe 2 (Fig. 3, lanes 2, 3, 5, 7), while two sublines produced the 700-kb fragments (Fig. 3, lanes 4 and 6). With probe 1, 400 kb fragments were detected in digests of DNA from all sublines (data not shown). These results show that the dAc-I-RS elements in cell line T/dAc#8 were transposed by 5 kb, 550 kb, and 200 kb toward the left border (LB) of the T-DNA, respectively, and no element transposed leftward to the right border (RB) within 400 kb (Fig. 4).

In the T/dAc#12 cell line, *Mlu*I sites nearest to the (I-RS/dAc-I-RS)T-DNA were mapped 700 kb and 600 kb toward RB and LB, respectively (Fig. 4). We also analyzed transposition events in this cell line using *Mlu*I. When we measured the sizes of *Mlu*I digests of genomic DNAs from ten sublines that were derived from the T/dAc#12 line and had transposed dAc-I-RS elements, only two sub-

Fig. 2. Analysis of PCR products after induction of dAc-I-RS excision by *Ac* transposase. Template DNAs were extracted from the T/dAc#8-K2 subline (column 1), the T/dAc#8-K3 subline (column 2), and the T/dAc#8 line (column 3). PCR was performed as described in Materials and Methods using primers (Fig. 1A) that depicted on the right. Amplified DNA products were fractionated on a 4% polyacrylamide gel. Numbers on the left indicate length (base pairs) of PCR products.

Table 4. Reinsertion frequency of dAc-I-RS element

Transformed lines	Number of Km ^r calli	Number of Km ^r and Hyg ^r calli (%)
T/dAc#8	8	7 (88)
T/dAc#12	17	13 (76)
T/dAc#13	18	6 (33)
T/dAc#19	7	4 (57)

Values in parentheses represent the percentages of Km^r and Hyg^r calli per Km^r calli.

lines produced DNA segments that were shorter in size than the detection limits described above. Fragments of 200 kb and 5 kb were detected by probe 1 and probe 2, respectively (data not shown). These results showed that the dAc-I-RS elements in the T/dAc#12 line were transposed by 200 kb and 5 kb toward the RB and LB of the T-DNA, respectively (Fig. 4).

We examined the orientation of the transposed dAc-I-RS elements at the insertion sites using probes that contained sequences specific for the left or the right side of the *Mlu*I site in dAc-I-RS. The results of these experiments showed that the dAc-I-RS element could be inserted in both possible orientations (data not shown).

Using the dAc-I-RS element that contained the *Mlu*I site, we examined the physical distances and directions of

Fig. 3. Southern blot analysis for measurement of dAc-I-RS transposition distance by pulse-field gel electrophoresis. High molecular weight genomic DNA was prepared from cultured cells of sublines that had a transposed dAc-I-RS that originated from cell line T/dAc#8, as described in Materials and Methods. Isolated DNA was digested with *Mlu*I, and then fractionated by PFGE in a 1% agarose gel for 18 hr at 170 volts with a switch interval of 80 sec, and then for 3 hr at 170 volts with a switch interval of 110 sec. After transfer of the DNA to a nylon membrane filter, the filter was probed with probe 2. Standards are shown with size in kilo-base pairs (kb). Lanes 1, the T/dAc#8 line (before transposition); lanes 2, 3, 4, 5, 6, and 7, T/dAc#8-K2, K3, K4, K6, K7, and K8 lines.

Fig. 4. Summary of the distances and directions of transposition in the T/dAc#8 and #12 lines. Cleavage sites for *Mlu*I in the tobacco genome are indicated by arrows.

Ac element transposition in two transformed BY-2 cell lines that had dAc-I-RS elements in different chromosomal locations. Seventy percent of the transposed dAc-I-RS elements were mapped within 700 kb on the chromosome in the T/dAc#8 cell line. In the T/dAc#12 cell line, 20% of the transposed dAc-I-RS elements were mapped within 200 kb on both sides of the original site of these elements. Our results showed that the Ac element can be efficiently transposed to sites within a few hundreds kb in tobacco cells. Although transposition frequency in tobacco is higher than in Arabidopsis plants (Baker et al., 1986), the pattern of transposition in tobacco cultured cells is similar to that in Arabidopsis (Machida et al., 1997), indicating that the pattern of transposition to linked chromosomal sites is characteristic of the Ac element, which is independent of the frequency of transposition. This result also suggests that the Ac element is useful for disrupting genes that are present in such adjacent regions, even in the absence of strong selective pressure. Interestingly, transposition events observed in the T/dAc#8 line were also detected in only one direction within 700 kb; transposition was not detected in the opposite direction within 400 kb. This is consistent with previously reported genetic analysis of asymmetric transposition (Greenblatt, 1984). However, we have to analyze more transposed lines to make such an asymmetric transposition on a chromosome more evident.

Use of the transposed dAc-I-RS in combination with the R-RS site specific recombination system. Development of recombinant DNA technology for manipulation of large segments of DNA is of importance for the structural and functional analysis of eukaryotic chromosomes. For development of this technology, the use of site-specific recombination systems, such as the cre-loxP system, the R-RS system and FLP-FRT systems, has been proposed (Onouchi et al., 1991; van Haaren and Ow, 1993; Machida et al., 1993; Medberry et al., 1995; Osborne et al., 1995; Onouchi et al., 1995). Chromosomal deletions and inversions have been induced in Arabidopsis, tobacco and rice plants by exploitation of these systems (Medberry et al., 1995; Osborne et al., 1995; Machida et al., 2000; Nakagawa et al., 2000b). However, the feasibility of exploiting this system has not been established, in particular for the induction of chromosomal deletions, because systematic analysis remains to be performed. One of the aims of our present study was to solve this problem. As shown in Fig. 1, the (I-RS/dAc-I-RS)T-DNA that we constructed contained two RS sequences, namely, recognition sites for a recombinase (R protein) from *Z. rouxii* (Machida et al., 1997). The tobacco cells that we generated in the present study will, therefore, be useful for a systematic examination of chromosomal deletions mediated by the R-RS system since the physical distance between two RS sequences on the chromosome have been precisely deter-

mined.

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